



FFW / AF
Appl. No. 10/688,845
Atty. Dkt. No. 076333-0393

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

Appellants: Michael T. Lotze *et al.*
Title: *METHODS AND REAGENTS FOR INDUCING IMMUNITY*
Appl. No.: 10/688,845
Filing Date: 10/15/03
Examiner: Amy E. Juedes
Art Unit: 1644
Confirmation Number: 9535

**BRIEF ON APPEAL AND RESPONSE TO
NOTIFICATION OF NON-COMPLIANT APPEAL BRIEF**

Mail Stop Appeal Brief - Patents
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Sir:

This appeal brief was originally filed on November 10, 2008, with the fee required by 37 C.F.R. 41.20(b)(2) and a Petition for Extension of Time. The Office issued a Notification of Non-Compliant Appeal Brief on December 5, 2008. The Notification stated that the Appeal Brief did not contain the Status of the Amendments and Related Proceedings Appendix sections.

This appeal brief is nearly identical to the November 10th brief except for changes made responsive to the December 5th Notification. Specifically, this brief contains sections entitled Status of the Amendments and Related Proceedings Appendix. The only other change is the inclusion of a section entitled "Related Appeals and Interferences" pursuant to 37 C.F.R. § 41.37(c)(ii).

No fees are believed to be due. But if any fees are necessary to timely file this Appeal Brief, authorization is hereby given to charge any deficiency (or credit any balance) to the undersigned deposit account 19-0741.

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REAL PARTY IN INTEREST

The real parties in interest are the University of Pittsburgh, the assignee of this application, and Intrexon Corp., a licensee.

RELATED APPEALS AND INTERFERENCES

Appellants are not aware of any prior or pending appeals, interferences, or judicial proceedings that may be related to, directly affect, or be directly affected by or have a bearing on the Board's decision in this appeal.

STATUS OF CLAIMS

Claims 1-31 and 35-40 are pending, and claims 1-26, 30 and 39 are withdrawn. Claims 32-34 were cancelled without prejudice or disclaimer.

Claims 27-29, 31, 35-38, and 40 stand rejected and are being appealed.

STATUS OF THE AMENDMENTS

No amendments were submitted following the Final Office Action dated April 10, 2008.

SUMMARY OF CLAIMED SUBJECT MATTER

Two independent claims, claims 27 and 36, are on appeal. Appellants provide below "a concise explanation of the subject matter defined in each of the independent claims involved in the appeal, referring to the specification by page and line number." *See* 37 C.F.R. § 41.37.

Claim 27 is directed to a therapeutic composition comprising an antigen presenting cell and an immunostimulatory cytokine or nucleic acid encoding an immunostimulatory cytokine. *See* specification at page 3, paragraph [0008] The antigen presenting cell is not loaded or pulsed with antigens. *Id.* at ¶ [0027]. The therapeutic composition also comprises a pharmaceutically acceptable solution or buffer. *Id.* at ¶ [0040]. The specification explains

that these therapeutic compositions can be used to treat tumor growth, metastasis, or infection. *Id.* at page 4, paragraph [0008].

Claim 36 is directed to a therapeutic composition comprising a pharmaceutically acceptable carrier, an antigen presenting cell, and an immunostimulatory cytokine or nucleic acid encoding an immunostimulatory cytokine. *Id.* at page 4, paragraph [0009]. The antigen presenting cell is not loaded or pulsed with antigens. *Id.* at ¶ [0027]. The specification explains that these formulations can be used to treat a subject having a tumor, metastasis, or infection lesion. *Id.* at page 16, paragraph [0042].

GROUND OF REJECTION TO BE REVIEWED ON APPEAL

This appeal presents two grounds of rejection for review:

(1) the rejection of claims 27-29, 31, 35-38, and 40 under 35 U.S.C. § 102(b), as allegedly anticipated by Bhardwaj *et al.*, J. CLIN. INVEST. 98:715-722 (1996), as evidenced by Hackstein *et al.*, BLOOD 100(3):1084-1087 (2002); and

(2) the rejection of claims 27-29, 31, 35-38, and 40 under 35 U.S.C. § 102(b), as allegedly anticipated by Kelleher *et al.*, INT'L IMMUNOLOGY 10(6):749-755 (1998).

ARGUMENT

The rejections should be withdrawn as both legally and factually flawed. The claims are directed to a “therapeutic composition.” In rejecting the claims, the Examiner disregards the preamble recitation of a “therapeutic composition.” Yet, in this instance the preamble breathes life and meaning into the claims, and Appellants relied on and continue to rely on the preamble to distinguish the prior art. Accordingly, the Examiner’s disregard of the preamble is legal error.

The Examiner also concludes that the prior art compositions, which are simply cell cultures, are “therapeutic compositions” because they are “physiologically compatible.” This conclusion is the manifestation of an unreasonable claim construction and is also contradicted by the evidence of record.

A. Preambles Can Limit Claim Scope

The determination of whether a preamble limits a claim is made on a case-by-case basis in light of the facts in each case. *Catalina Mktg. Int'l v. Coolsavings.com, Inc.*, 289 F.3d 801, 808, 62 USPQ2d 1781, 1785 (Fed. Cir. 2002). In performing an analysis, the entire claim should be considered along with the specification to determine what the inventors intended to be encompassed by the claim. *Corning Glass Works v. Sumitomo Electric U.S.A., Inc.*, 868 F.2d 1251, 1257 (Fed. Cir. 1989); *Pitney Bowes, Inc. v. Hewlett-Packard Co.*, 182 F.3d 1298, 1305, 51 USPQ2d 1161, 1165-66 (Fed. Cir. 1999).

The preamble should be limiting if it is “necessary to give life, meaning, and vitality” to the claim when read as a whole. *Pitney Bowes, Inc. v. Hewlett-Packard Co.*, 182 F.3d 1298, 1305 (Fed. Cir. 1999) (quotations omitted) (citations omitted). The repeated reference to the preamble in the specification and an indication that it constitutes an important characteristic of the invention is sufficient to make the preamble limiting. *Poly-America, L.P. v. GSE Lining Tech., Inc.*, 383 F.3d 1303, 1310 (Fed. Cir. 2004).

“[C]lear reliance on the preamble during prosecution to distinguish the claimed invention from the prior art transforms the preamble into a claim limitation because such reliance indicates use of the preamble to define, in part, the claimed invention.” *Catalina Mktg. Int'l v. Coolsavings.com, Inc.*, 289 F.3d 801, 808 (Fed. Cir. 2002). The reliance can take the form of amending the claims to add the limitation. *Invitrogen Corp. v. Biocrest Mfg., L.P.*, 327 F.3d 1364, 1370 (Fed. Cir. 2003). The reliance can also be based on arguments distinguishing the prior art based on the preamble. *In re Cruciferous Sprout Litig.*, 301 F.3d 1343, 1348 (Fed. Cir. 2002).

B. “Therapeutic Composition” Serves As A Limitation (Claims 27-29, 31, 35-38 and 40)

When read on the proper context, one of skill in art would understand the term “therapeutic composition” to breath life and meaning into the claims. The specification is replete with references to the therapeutic importance of the claimed invention. Indeed, the Field of the Invention section states that “the invention is directed to the use of co-administration of antigen presenting cells and immunostimulatory cytokines *to treat tumors*

or infections.” Spec. at ¶ [0003] (emphasis added). Consistent with the Field of the Invention section, the Summary of the Invention section states that the “instant invention relates to methods and reagents *for treating a tumor or infection* by administering an immunostimulatory cytokine, or a nucleic acid encoding an immunostimulatory cytokine, in combination with antigen presenting cells *into or near a tumor or infectious lesion.*” Spec. at ¶ [0007] (emphasis added). The remaining portions of the specification provide details on the therapeutic composition and how they can be administered to “treat[] a tumor or infection.” Thus, the specification leaves no doubt. The invention is not directed to mere cultures. Rather, the invention is directed to, *inter alia*, therapeutic compositions “to treat tumors or infections.”

The claim language itself emphasizes that the “therapeutic composition” language breathes life and meaning into the claims. Claims 27 and 36 recite “a physiologically acceptable solution or buffer” and “a pharmaceutically acceptable carrier,” respectively. Unless the claims were directed to “therapeutic compositions,” there would be no reason for this language. Cell cultures do not need to include physiologically acceptable solutions or buffers or pharmaceutical carriers. This language only makes sense when considered with the “therapeutic composition” preamble. Thus, the claims themselves, when considered as a whole, confirm that the “therapeutic composition” language is intended to breath life and meaning into the claims. *Aventis Pharms., Inc. v. Barr Labs., Inc.*, 341 F. Supp. 2d 502, 509 (D.N.J. 2004) (holding “pharmaceutical composition” in preamble to be limiting).

Moreover, Appellants have repeatedly relied on the “therapeutic composition” language to distinguish the prior art, which discloses cell cultures with no suggestion of therapeutic applicability. Accordingly, Appellants have defined the claimed invention, in part, based on the preamble. Accordingly, the preamble constitutes a claim limitation. *Catalina Mktg. Int’l v. Coolsavings.com, Inc.*, 289 F.3d 801, 808 (Fed. Cir. 2002); *In re Cruciferous Sprout Litig.*, 301 F.3d 1343, 1348 (Fed. Cir. 2002).

C. The Prior Art Does Not Teach The Claimed “Therapeutic Composition”

1. The Prior Art Teaches Cell Cultures Rather Than Therapeutic Compositions

It is undisputed that the Bhardwaj and Kelleher do not teach or suggest a therapeutic application. Instead, Bhardwaj and Kelleher disclose cell cultures developed in the course of general scientific research. Specifically, Bhardwaj discloses a cell culture system that has both IL-12 and dendritic cells, which are either infected with influenza or uninfected. These compositions were used to evaluate the role of IL-12 in generating cytolytic T lymphocyte (CTL) responses to influenza virus. Kelleher discloses a cell culture system comprising dendritic cells. IL-12 was added to some of these cultures to determine “whether IL-12 administration during DC maturation altered cell numbers, phenotype and function.” Kelleher at 749, right col. Neither of these references discloses a “therapeutic composition,” as claimed.

One of skill in the art would understand a “therapeutic composition” as follows:

a composition suitable for administration to a patient for the treatment of some disease or condition. Therapeutic compositions are formulated so as to preserve the stability of the active agents while making the composition biologically compatible. Thus, a therapeutic composition is more than just an active agent. A therapeutic composition contains one or more active agents formulated such that they can be safely administered to patients for the treatment of some disease or condition.

Declaration Under 37 C.F.R. § 1.132 of Michael T. Lotze (“Lotze Decl.”), submitted June 29, 2007, at ¶ 7. Bhardwaj and Kelleher do not teach suggest a composition. Rather, the references teach cell cultures designed to explore the role of IL-12. Nothing in Bhardwaj and Kelleher teaches or suggests that the cultures have any therapeutic value, much less that they should be formulated to be safely administered to patients. Accordingly, Bhardwaj and Kelleher do not teach or suggest the claimed invention.

2. The Prior Art Cultures Are Incompatible With “Physiological Composition”

The Examiner contends that the cultures of Bhardwaj and Kelleher constitute “therapeutic compositions” because “[c]ells in culture are considered to be compatible with physiological conditions an not incompatible with therapeutical use.” Office Action dated April 10, 2008 at ¶ 5. This contention fails for two reasons.

First, the contention is premised on a legally flawed claim construction. The broadest reasonable interpretation of claims must be used during examination, but “[t]he broadest reasonable interpretation of the claims must also be consistent with the interpretation that those skilled in the art would reach.” MPEP § 2111. The broadest reasonable interpretation is based on the “ordinary and customary meaning” claim terms would have to one of ordinary to skill in the art. MPEP § 2111.01(II).

One of skill in the art understands that a “therapeutic composition” is a composition suitable for administration to a patient for the treatment of some disease or condition and contain an active agent along with addition components to preserve the active of the active agent. Lotze Decl. at ¶¶ 7, 9. Thus, even if cell culture may be “physiologically compatible,” one of skill in the art would not necessarily understand that culture to be a “therapeutic composition.” In order to be a “therapeutic composition,” there must be some indication that the composition should actually be used in that manner (i.e. administered to a patient). *Id.* Accordingly, the broadest reasonable construction of “therapeutic composition” requires something more than mere physiological compatibility.

Second, even if a “therapeutic composition” could be reasonably construed to be any physiologically compatible composition, the prior art does not disclose a physiologically compatible composition.

Bhardwaj’s cell culture is not “physiologically compatible” because Bhardwaj’s crude culture contains agents, such as impurities, antithetical to a “therapeutic composition.” Lotze Decl. at ¶ 10. Moreover, Bhadowaj’s cell cultures are “infected with a live or heat-inactivated influenza virus.” Bhardwaj, page 716, left column. Such a composition could cause serious

complications if it were to be administered to a patient. Lotze Decl. at ¶ 10. Accordingly, one of skill in the art would consider Bhardwaj's cell culture to be unsuitable for use as a therapeutic agent. *Id.*

Nor is Kelleher's culture "physiologically compatible," because Kelleher's culture contains, "RPMI 1640 (Dutch modification; Sigma, Poole, UK) supplemented with 10% FCS (Gibco, Paisley, UK), 100 U Penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 2 mM glutamine (ICN Flow, Irvine, UK), 2 mercaptoethanol 10^{-5} M (Sigma)." Kelleher, p. 750. One of skill in the art would understand that administration of several components Kelleher's culture, such as FCS (fetal calf serum) and 2-mercaptoethanol, could cause serious complications if it were to administration to a patient. Lotze Decl. at ¶ 10. Accordingly, the cultures of Bhardwaj and Helleher are not "physiologically compatible" as "therapeutic components."

D. Conclusion

Appellants respectfully request that the rejections of claims 27-29, 31, 35-38 and 40 be reversed because the rejections are unsupported by the law or the evidence of record.

Respectfully submitted,

Date January 5, 2009

By 

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CLAIMS APPENDIX

1. (Withdrawn) A method of inhibiting or treating a tumor or infectious lesion in a subject, comprising: administering into or near a site of a tumor or infectious lesion in a subject an effective amount of an antigen presenting cell and an immunostimulatory cytokine or a nucleic acid encoding an immunostimulatory cytokine.

2. (Withdrawn) The method of claim 1, wherein the antigen presenting cell is a dendritic cell.

3. (Withdrawn) The method of claim 2, wherein the dendritic cell is selected from the group consisting of a CD34+-derived dendritic cell, a bone marrow-derived dendritic cell, a monocytederived dendritic cell, a splenocyte derived dendritic cell, a skin-derived dendritic cell, a follicular dendritic cell, and a germinal center dendritic cell.

4. (Withdrawn) The method of claim 1, wherein the dendritic cell is a CD34+-derived dendritic cell cultured in the presence of at least one factor selected from the group consisting of granulocyte colony stimulating factor, granulocyte macrophage colony stimulatory factor, tumor necrosis factor alpha, interleukin 4, the Flt-3 ligand, and the kit ligand.

5. (Withdrawn) The method of claim 1, wherein the antigen presenting cell is selected from a group consisting of a Langherhans' cell, an interdigitating cell, a B cell, and a macrophage.

6. (Withdrawn) The method of claim 1, wherein the immunostimulatory cytokine is selected from the group consisting of interleukin-1 α ., interleukin-1 β , interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-8, interleukin-9, interleukin-10, interleukin-12, interleukin-18, interleukin-19, interleukin-20, interleukin-23, interleukin-27, interleukin-1f3, interleukin-1f5, interleukin-1f6, interleukin-1f7, interleukin-1f8, interleukin-1f9, interleukin-1f10, interferon- α ., interferon- β , interferon- γ , tumor necrosis factor α , transforming growth factor- β ,

granulocyte colony stimulating factor, macrophage colony stimulating factor, granulocyte-macrophage colony stimulating factor, the Flt3 ligand, and the kit ligand.

7. (Withdrawn) The method of claim 1, wherein the expression vector is a viral vector.

8. (Withdrawn) The method of claim 2, wherein the expression vector is selected from the group consisting of an adenoviral vector, an adeno-associated viral vector, a retroviral vector, a lentiviral vector, a herpes viral vector, and a vaccinia viral vector.

9. (Withdrawn) The method of claim 1, wherein the subject has a tumor selected from the group consisting of melanoma, hepatoma, adenocarcinoma, colorectal cancer, basal cell cancer, oral cancer, nasopharyngeal cancer, laryngeal cancer, bladder cancer, head and neck cancer, renal cell cancer, pancreatic cancer, pulmonary cancer, cervical cancer, ovarian cancer, esophageal cancer, gastric cancer, prostate cancer, testicular cancer, and breast cancer.

10. (Withdrawn) The method of claim 1, wherein the size of the tumor or infectious lesion is decreased.

11. (Withdrawn) The method of claim 1, wherein said administering step comprises injecting into the tumor or infectious lesion.

12. (Withdrawn) The method of claim 1, wherein said administering step comprises injecting the subject within the same organ as the tumor or infectious lesion.

13. (Withdrawn) A method of inhibiting or treating metastasis of a tumor in a subject, comprising: administer into or near a site of a tumor in a subject an effective amount of an antigen presenting cell and an immunostimulatory cytokine or a nucleic acid encoding an immunostimulatory cytokine.

14. (Withdrawn) The method of claim 13, wherein the antigen presenting cell is a

dendritic cell.

15. (Withdrawn) The method of claim 14, wherein the dendritic cell is selected from the group consisting of a CD34+-derived dendritic cell, a bone marrow-derived dendritic cell, a monocyte-derived dendritic cell, a splenocyte derived dendritic cell, a skin-derived dendritic cell, a follicular dendritic cell, and a germinal center dendritic cell.

16. (Withdrawn) The method of claim 13, wherein the dendritic cell is a CD34+-derived dendritic cell cultured in the presence of at least one factor selected from the group consisting of granulocyte colony stimulating factor, granulocyte macrophage colony stimulatory factor, tumor necrosis factor alpha, interleukin 4, the Flt-3 ligand, and the kit ligand.

17. (Withdrawn) The method of claim 13, wherein the antigen presenting cell is selected from a group consisting of a Langherhans' cell, an interdigitating cell, a B cell, and a macrophage.

18. (Withdrawn) The method of claim 13, wherein the immunostimulatory cytokine is selected from the group consisting of interleukin-1 α , interleukin-1 β , interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-8, interleukin-9, interleukin-10, interleukin-12, interleukin-18, interleukin-19, interleukin-20, interleukin-23, interleukin-27, interleukin-1f3, interleukin-1f5, interleukin-1f6, interleukin-1f7, interleukin-1f8, interleukin-1f9, interleukin-1f10, interferon- α , interferon- β , interferon- γ , tumor necrosis factor α , transforming growth factor- β , granulocyte colony stimulating factor, macrophage colony stimulating factor, granulocyte-macrophage colony stimulating factor, the Flt3 ligand, and the kit ligand.

19. (Withdrawn) The method of claim 13, wherein the expression vector is a viral vector.

20. (Withdrawn) The method of claim 13, wherein the expression vector is selected from the group consisting of an adenoviral vector, an adeno-associated viral vector, a retroviral vector, a lentiviral vector, a herpes viral vector, and a vaccinia viral vector.

21. (Withdrawn) The method of claim 13, wherein the subject has a tumor selected from the group consisting of melanoma, hepatoma, adenocarcinoma, colorectal cancer, basal cell cancer, oral cancer, nasopharyngeal cancer, laryngeal cancer, bladder cancer, head and neck cancer, renal cell cancer, pancreatic cancer, pulmonary cancer, cervical cancer, ovarian cancer, esophageal cancer, gastric cancer, prostate cancer, testicular cancer, and breast cancer.

22. (Withdrawn) The method of claim 13, wherein the size of the tumor or infectious lesion is decreased.

23. (Withdrawn) The method of claim 13, wherein the size of the metastasis is decreased.

24. (Withdrawn) The method of claim 13, wherein the number of the metastases is

25. (Withdrawn) the method of claim 13, wherein said administering step comprises injecting into the tumor or infectious lesion.

26. (Withdrawn) The method of claim 13, wherein said administering step comprises injecting the subject within the same organ as the tumor or infectious lesion.

27. (Previously Presented) A therapeutic composition comprising (a) a physiologically acceptable solution or buffer, (b) an antigen presenting cell, and (c) an immunostimulatory cytokine or a nucleic acid encoding an immunostimulatory cytokine, wherein the antigen presenting cell is not loaded or pulsed with antigens.

28. (Previously Presented) The composition of claim 27, wherein the antigen presenting cell is a dendritic cell.

29. (Previously Presented) The composition of claim 28, wherein the dendritic cell is selected from the group consisting of a CD34+-derived dendritic cell, a bone marrow-derived dendritic cell, a monocyte-derived dendritic cell, a splenocyte derived dendritic cell, a skin-derived dendritic cell, a follicular dendritic cell, and a germinal center dendritic cell.

30. (Withdrawn) The composition of claim 27, wherein the antigen presenting cell is selected from a group consisting of a Langherhans' cell, an interdigitating cell, a B cell, and a macrophage.

31. (Previously Presented) The composition of claim 27, wherein the immunostimulatory cytokine is selected from the group consisting of interleukin- α , interleukin-1 β , interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-8, interleukin-9, interleukin-10, interleukin-12, interleukin-18, interleukin-19, interleukin-20, interleukin-23, interleukin-27, interleukin-1f3, interleukin-1f5, interleukin-1f6, interleukin-1f7, interleukin-1f8, interleukin-1f9, interleukin-1f10, interferon- α , interferon- β , interferon- γ , tumor necrosis factor α , transforming growth factor- β , granulocyte colony stimulating factor, macrophage colony stimulating factor, granulocyte-macrophage colony stimulating factor, the Flt-3 ligand, and the kit ligand.

32.-34. (Canceled).

35. (Previously Presented) The composition of claim 27, further comprising a carrier.

36. (Previously Presented) A therapeutic composition comprising (a) a pharmaceutically acceptable carrier, (b) an antigen presenting cell, and (c) an immunostimulatory cytokine or a nucleic acid encoding an immunostimulatory cytokine, wherein the antigen presenting cell is not loaded or pulsed with antigens.

37. (Previously Presented) The composition of claim 36, wherein the antigen presenting cell is a dendritic cell.

38. (Previously Presented) The composition of claim 37, wherein the dendritic cell is selected from the group consisting of a CD34+-derived dendritic cell, a bone marrow-derived dendritic cell, a monocyte-derived dendritic cell, a splenocyte derived dendritic cell, a skin-derived dendritic cell, a follicular dendritic cell, and a germinal center dendritic cell.

39. (Previously Presented) The composition of claim 36, wherein the antigen presenting cell is selected from a group consisting of a Langherhans' cell, an interdigitating cell, a B cell, and a macrophage.

40. (Previously Presented) The composition of claim 36, wherein the immunostimulatory cytokine is selected from the group consisting of interleukin-1 α , interleukin-1 β , interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-8, interleukin-9, interleukin-10, interleukin-12, interleukin-18; interleukin-19, interleukin-20, interleukin-23, interleukin-27, interleukin-1f3, interleukin-1f5, interleukin-1f6, interleukin-1f7, interleukin-1f8, interleukin-1f9, interleukin-1f10, interferon- α , interferon- β , interferon- γ , tumor necrosis factor α , transforming growth factor- β , granulocyte colony stimulating factor, macrophage colony stimulating factor, granulocyte-macrophage colony stimulating factor, the Flt3 ligand, and the kit ligand.

EVIDENCE APPENDIX

Declaration Under 37 C.F.R. § 1.132 of Michael T. Lotze submitted June 29, 2007.

Bhardwaj *et al.*, J. CLIN. INVEST. 98:715-722 (1996).

Hackstein *et al.*, BLOOD 100(3):1084-1087 (2002).

Kelleher *et al.* INT'L IMMUNOLOGY 10(6):749-755 (1998).

RELATED PROCEEDINGS APPENDIX

Because Appellants are not aware of any related appeals, judicial proceedings, or interferences, there are not copies of any decisions to submit.



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Michael T. LOTZE et al.
Title: METHODS AND REAGENTS FOR INDUCING IMMUNITY
Appl. No.: 10/688,845
Filing Date: 10/15/2003
Examiner: Amy E. Juedes
Art Unit: 1644
Confirmation Number: 9535

DECLARATION UNDER 37 C.F.R. § 1.132 OF MICHAEL T. LOTZE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Michael T. Lotze, declare as follows:

1. I am one of the inventors named on the captioned application.
2. My education includes a B.Med. Sciences, as well as an M.D., from Northwestern University and its Medical School.
3. I am a board licensed physician with board certifications in surgery and surgical oncology. Currently, I am Professor of Surgery and Bioengineering; Associate Director UPCI for Strategic Partnerships; Vice Chair of Research in the Department of Surgery; and Assistant Vice Chancellor for Sponsored Training Grants, University of Pittsburgh Schools of the Health Sciences.
4. In addition to my clinical duties, I perform research regarding the role of the immune system in cancer and the potential for using immune responses to treat cancer. I am

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currently most interested in using natural killer (NK) cells and dendritic cells for cancer therapy. We have a National Cancer Institute funded Program Project Grant in this area.

5. My curriculum vitae is attached as Exhibit A.

6. I have read and understand the Office Action dated March 7, 2007 regarding the captioned application. I understand that the Office Action contends that claims 27-29 and 31-35 are anticipated by Bhardwaj *et al.*, J. CLIN. INVEST. 98:715-722 (1996) as evidenced by Hackstein *et al.*, BLOOD 100(3):1084-1087 (2002) and Kelleher *et al.* INT'L IMMUNOLOGY 10(6):749-755 (1998). I disagree.

7. Physicians and medical researchers understand a "therapeutic composition" to be a composition suitable for administration to a patient for the treatment of some disease or condition. Therapeutic compositions are formulated so as to preserve the stability of the active agents while making the composition biologically compatible. Thus, a therapeutic composition is more than just an active agent. A therapeutic composition contains one or more active agents formulated such that they can be safely administered to patients for the treatment of some disease or condition.

8. Turning to the references cited by the Office Action, Bhardwaj and Kelleher disclose cell cultures generated as part of research. More specifically, Bhardwaj discloses a cell culture that has both IL-12 and dendritic cells, which are either infected with influenza or uninfected. These compositions were used to evaluate the role of IL-12 in generating cytolytic T lymphocyte (CTL) responses to influenza virus. Kelleher discloses cultures comprising dendritic cells. IL-12 was added to some of these cultures to determine "whether IL-12 administration during DC maturation altered cell numbers, phenotype and function." Kelleher at 749, right col. The dendritic cells were cultured in "RPMI 1640 (Dutch modification; Sigma, Poole, UK) supplemented with 10% FCS (Gibco, Paisley, UK), 100 U Penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 2 mM glutamine (ICN Flow, Irvine, UK), 2 mercaptoethanol 10⁻⁵ M (Sigma)." *Id.*

9. Physicians and medical researchers would not understand Bhardwaj and Kelleher to disclose a "therapeutic composition." Bhardwaj and Kelleher disclose cell

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cultures, as I discussed above. Nothing in these references suggests that the cell cultures have any therapeutic value. The cultures were simply used to investigate the role of IL-12. Accordingly, a physician reading Bhardwaj and Kelleher would not consider their cell cultures to be "therapeutic compositions."

10. In addition, one of skill in the art would not consider the cultures of Bhardwaj and Kelleher to be "compatible with physiological conditions." These raw cell cultures would contain contaminants and impurities that may cause potentially serious reactions in patients. Many components of raw cell cultures have inhibitory proteins that are known, such as IL10 or TGF β , or other elements which are unknown. Accordingly, a physician would not consider the cell cultures of Bhardwaj and Kelleher to be suitable for use "therapeutic composition[s]."

* * * * *

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: June 29, 2007

By: 

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IL-12 in Conjunction with Dendritic Cells Enhances Antiviral CD8+ CTL Responses In Vitro

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Abstract

CD8+ cytolytic T lymphocytes (CTLs) are important mediators for resistance to infections and malignant diseases. IL-12 enhances proliferative and cytolytic responses by killer cells, but its function in the generation of human antiviral CD8+ T cell responses has not been defined. We therefore evaluated the role of IL-12 in the generation of CTLs to influenza-infected dendritic cells. IL-12 was not detectable in supernatants of infected-dendritic cells, or during CTL generation. Furthermore, anti-IL-12 antibody did not block CTL generation. However, exogenous IL-12 (30–300 pg/ml) enhanced CD8+ T cell proliferative and cytolytic responses. The effect was greatest in individuals with weak reactivity to influenza virus or at antigen-presenting cell (APC):T cell ratios of 1:100 or less. IL-12 augmented interferon- γ production during CTL generation. The CTL enhancing effects of the cytokine, however, could not be blocked by neutralizing anti-interferon- γ antibody. Together with IL-12, antigen-pulsed dendritic cells may be a useful approach for boosting CTL responses against infectious agents and malignancies. (*J. Clin. Invest.* 1996; 98: 715–722.) Key words: dendritic cells • cytolytic T cells • CD8+ T cells • interleukin 12 • vaccines

Introduction

IL-12 is a heterodimeric cytokine with multiple immunoregulatory activities (1, 2). A critical component of the host's innate immune response to infection, IL-12 is produced early during the inflammatory response by macrophages. It enhances natural killer (NK)¹ cell cytotoxicity severalfold and rapidly induces the production of IFN γ , the latter enhancing the antimicrobial activity of phagocytic cells (3–6). IL-12 is also a crucial participant in the development of acquired immune responses. It induces the differentiation of Th1 cells through its ability to prime naive Th0 cells for high IFN γ production (7, 8). Furthermore, it is a potent growth factor for CD8+ T cells (7) and enhances cytolytic responses to alloantigens (9–11) and in anti-CD3 redirected assays (12).

Here, we have evaluated the role of IL-12 in generating cy-

tolytic T lymphocyte (CTL) responses to influenza virus, which continues to be a source of significant morbidity and mortality in humans. We have previously shown that potent human CD8+ CTL responses to live or nonreplicating heat-inactivated influenza virus can be generated from freshly isolated blood T cells, provided dendritic cells are the antigen-presenting cells (APCs) (13, 14). The response is independent of CD4+ T cells or exogenous cytokines. Small numbers of dendritic cells suffice, whereas other APCs (macrophages, B cells) are inactive in this regard. Dendritic cells effect the development of Th1 cells from Th0 cells by the production of IL-12 (15), suggesting that IL-12 is a key component by which these APCs induce T cell-mediated immune responses.

Using the influenza virus system as a model, we show that dendritic cells induce potent CTL responses without producing significant levels of IL-12 endogenously. However, the exogenous application of IL-12 significantly enhances these responses, especially when the baseline CTL response of the donor is poor, or if the number of dendritic cells is limiting. Based on these findings we suggest that IL-12 may be a useful adjuvant for the induction of CTL responses, especially when delivered concomitantly with dendritic cells.

Methods

Culture medium

RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 20 μ g/ml gentamicin, 5% human serum, and 10 mM Hepes buffer.

Blood mononuclear cells

T cells. PBMCs were isolated from buffy coats by density gradient centrifugation over Ficoll Hypaque. T cell-enriched and T cell-depleted (ER-) fractions were prepared by sheep erythrocyte rosetting. T cells were further purified by removal of monocytes, NK cells, and MHC class II+ cells by panning with antibodies to CD11b, CD16, and DR, as described (13). T cell subsets (CD4+ and CD8+) were negatively selected by incubation with Leu 2 or Leu 3 mAbs, followed by panning onto plastic plates coated with goat anti-mouse IgG (13).

Antigen presenting cells. Monocytes were obtained from ER-cells by adherence to plastic for 90 min. The monocytes were dislodged and used as targets in ⁵¹Cr release assays (see below). Nonadherent ER- cells were depleted of residual monocytes by panning on gamma globulin-coated dishes. The remaining cells (primarily B cells and dendritic cells) were adequately enriched for dendritic cells to induce strong CTL responses (13). Since B cells pulsed with influenza virus do not elicit CTL responses, in part because the B cell is not infected with influenza virus (13), we used mixtures of B cells and dendritic cells as APCs in some experiments. Highly purified dendritic cells (> 75%) were obtained as low density cells by metrizamide gradient centrifugation, as previously described (13).

Virus preparation

Influenza A virus (PR8, Puerto Rico/8/34, source: allantoic fluid; Spafas Inc., Storrs, CT) was either live, or inactivated at 56°C for 30 min in a water bath. Nonreplicating, heat-inactivated virus, which re-

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1. Abbreviations used in this paper: APC, antigen-presenting cell; CTL, cytolytic T lymphocyte; E:T, effector:target; NK, natural killer.

tains hemolytic and hemagglutinating activity, is as potent as live virus in inducing CD8⁺ antiinfluenza CTL responses (14).

Cytokines

IL-12 (specific activity 5×10^6 U/mg) was generously provided by Dr. Stanley Wolf (Immunology Department, Genetics Institute, Inc.). Recombinant human IL-4, IL-15, and IL-7 were purchased from R & D Systems Inc. (Minneapolis, MN). ELISA kits measuring IL-12, IL-2, IL-6, TNF α , and IFN γ were purchased from R & D Systems, Inc.

Neutralizing antibodies

Polyclonal goat anti-human IL-12, IL-2R (alpha chain, CD25), and IFN γ antibodies were purchased from R & D Systems, Inc. The hybridoma producing neutralizing anti-human IL-12 monoclonal antibody (C8.6) was kindly provided by Dr. G. Trinchieri of the Wistar Institute (Philadelphia, PA).

Induction of CTL responses

Dendritic cells were infected with 1,000 HAU/ml (moi of 2–4) of different forms of live or heat-inactivated influenza virus for 1 h at 37°C in serum free medium, washed extensively, and added to bulk cultures of purified syngeneic T cells in 24-well or 48-well plastic dishes (Falcon Labware, Lincoln Park, NJ). After 7 d, the cells were harvested and distributed in varying numbers in 100- μ l vol to 96-well microtiter plates. CTL activity was measured using a standard 51 Cr-release assay with infected or uninfected macrophages, as previously described (13). Effector:target (E:T) ratios were 30:1–40:1. In brief, macrophages were brought to 10^7 /ml in serum-free medium, and infected with 1,000 HAU/ml of influenza virus. The cells were simultaneously labeled with 51 Cr by the addition of 400 μ Ci of Na 51 CrO $_4$ (1 mCi/ml, sterile stock; New England Nuclear, Boston, MA) per milliliter. The targets were then washed four times, resuspended to 2×10^5 /ml, and aliquoted in 50 μ l vol to 96-well round bottom dishes containing effector cells. Percent specific 51 Cr release was calculated from the following formula: $100 \times ([\text{release by CTL} - \text{spontaneous release}] / [\text{total release} - \text{spontaneous release}])$. Spontaneous release was generally 15% of the total release.

Proliferation assays

2×10^5 bulk or CD8⁺ T cells were plated in 96-well flat bottomed plates (Costar, Cambridge, MA) in the presence or absence of varying concentrations of cytokines. APCs were partially purified dendritic cells (T:APC ratio of 5:1) or purified dendritic cells (T:APC ratio of 30:1). APCs were infected with influenza virus as described above. Uninfected APCs served as controls. Proliferation was determined on days 5–6 with the addition of 4 μ Ci/ml of 3 H-TdR for 12–16 h to triplicate microwells (mean cpm).

Results

IL-12 is not produced in dendritic cell-dependent CTL responses. Potent antiinfluenza CTL responses are induced from resting CD8⁺ T cells when dendritic cells are the APCs (13). Dendritic cells can produce IL-12 (15, 16) and direct the development of Th1 cells from CD4⁺ T cells (15). However, it is not known whether IL-12 is synthesized after infection with virus and during contact with CD8⁺ T cells. Using a sensitive ELISA system, we found that dendritic cells infected with live replicating influenza virus produced little to no IL-12 p70 heterodimer (range of 4–10 pg/ml in four separate experiments). Furthermore, significant levels of IL-12 were not detectable in T cell–dendritic cell culture supernatants over the 7-d period during which CD8⁺ CTLs normally develop (< 15 pg/ml). In contrast the cytokines IL-2, TNF α , IFN γ and IL-6 were detectable by ELISA (data not shown). Several other stimuli including LPS (reported to induce IL-12 mRNA in murine splenic

dendritic cells, [17]), superantigens (staphylococcal enterotoxins), or addition of allogeneic T cells also failed to elicit high levels of IL-12 by dendritic cells.

In the CTL cultures, dendritic cells were added at 1 cell per 30 T cells. We considered the possibility that very low levels of IL-12 were produced in the supernatants. Neutralizing anti-IL-12 antibody was therefore added to the culture system. CTL responses were not blocked by anti-IL-12 antibody (5–25 μ g/ml [Fig. 1] and data not shown) but were significantly suppressed by the addition of anti-IL-2 R antibody (α chain specific). IFN γ production was also unaffected by the addition of anti-IL-12 to the dendritic cell–T cell cultures. This suggests that endogenous IL-2, rather than IL-12, was responsible for IFN γ production. Indeed, IFN γ levels were markedly diminished by the addition of anti-IL-2 R α antibody (Fig. 1). Thus our data suggest that dendritic cells do not produce biologically significant levels of IL-12 after virus infection, and that IL-12 is not produced during the development of potent antiviral CTLs. The antiinfluenza CTLs that are generated in bulk T cell cultures by virus-infected dendritic cells are CD8⁺ (13). However, there is a substantial number of CD4⁺ T cells presumably responding to influenza antigens in a class II restricted fashion. Even their presence, however, was insufficient to elicit IL-12 from influenza infected dendritic cells.

IL-12 enhances T cell proliferative responses to influenza-virus infected dendritic cells. IL-12 stimulates the proliferation of activated T and NK cells but causes minimal proliferation of resting peripheral blood mononuclear cells (7). We evaluated the effects of IL-12 on enhancing T cell proliferative responses to influenza virus-infected dendritic cells. Marked increases in proliferative responses were noted with the addition of IL-12 at low doses (Fig. 2). IL-12 is known to enhance proliferative responses to mitogen-activated T cells in the picomolar range (7). This was true for both bulk T cells (Fig. 2A),

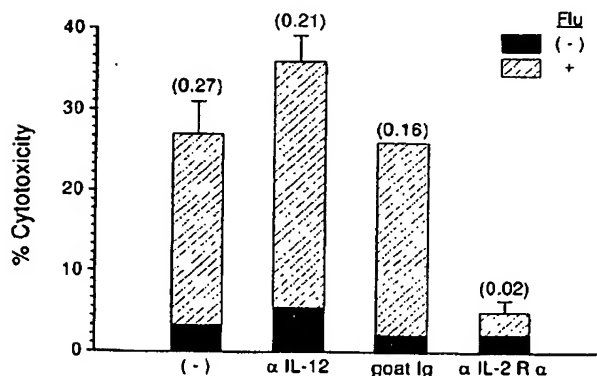


Figure 1. Antiviral CTL and IFN γ responses that are induced by dendritic cells are blocked by anti-IL-2 but not anti-IL-12 antibodies. Bulk T cells were cocultured with uninfected or influenza infected dendritic cells (T:DC ratio 30:1) in the presence of 5 μ g/ml of goat anti-IL-12 ab and anti-IL-2R α chain ab, or goat IgG. After 7 d the effector cells were tested for cytolytic activity of influenza infected syngeneic macrophage targets in a Cr release assay. E:T ratio was 30:1. Lysis of uninfected macrophage targets was $< 5\%$ (data not shown). Similar results were obtained in three other experiments. The numbers in parentheses refer to the concentrations of IFN γ (ng/ml) in the supernatants on the third day of culture.

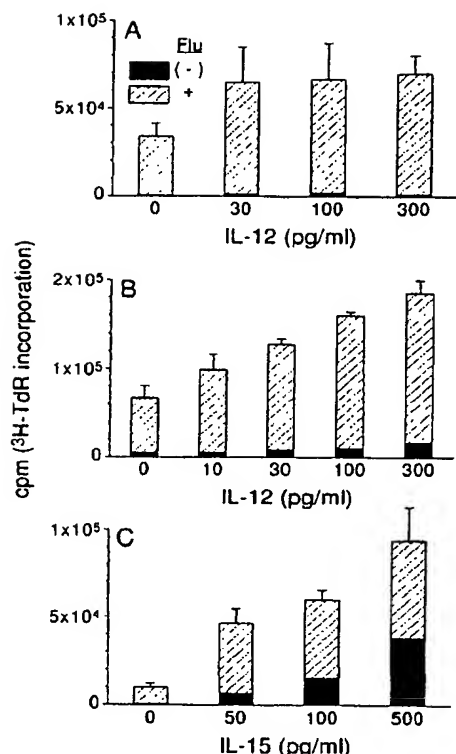


Figure 2. IL-12 enhances T cell proliferative responses to influenza virus-infected dendritic cells. Bulk T cells (A). (C) or CD8+ T cells (B), were cultured with virus-infected or uninfected APCs for 5 d. IL-12 (A and B) and IL-15 (C) were added in graded doses. Black portions of the bars represent the proliferative response to uninfected APCs while the hatched portion represent the responses to influenza-infected APCs. Proliferation was determined by the addition of ³H-TdR for 16 h on day 5. Results are means of triplicates. The data are representative of two experiments.

as well as for highly purified CD8+ T cells (Fig. 2 B). Background responses were minimal. T cell yields were increased up to twofold (data not shown). We also evaluated IL-15, a cytokine which shares many of the properties of IL-2 (18), and enhances CD4+ T cell-dependent responses to antigen (19). In contrast to IL-12, IL-15 produced dramatic background responses in bulk T cell populations (Fig. 2 C). Antigen specific T cell proliferative responses were still evident, however. IL-15 can induce some proliferation in bulk populations of human blood mononuclear cells (19). When IL-15 was added to purified CD8+ T cells, the responses generated to uninfected vs virus-infected dendritic cells was similar even with low doses, e.g., 50 pg/ml (data not shown).

IL-12 enhances weak antiviral cytolytic T cell responses. While dendritic cells induce strong CD8+ CTL responses to influenza virus, macrophages are weak or inactive. This may be due to apoptosis, observed 12–16 h after infection of macrophages with virus (20). However, macrophages are useful as target cells in short term CTL assays because > 70% get infected. B cells are poor stimulators of the T cell response to the virus, probably because they are infected poorly with influenza (13). As a result it is convenient for eliciting CTL responses to select macrophages as CTL targets, and to use T and macrophage-depleted blood populations as partially enriched sources of dendritic cells. The latter generally contain about 3–5% dendritic cells and are highly effective at inducing CD8+ CTL responses (13).

IL-12 (100 pg/ml) enhanced the cytolytic activity of bulk T cells towards influenza-infected, macrophage target cells (Fig. 3). Two situations were identified where IL-12 was most effective. When low doses of APCs were used (T:APC ratio = 30:1, Fig. 3A or T:dendritic cell ratio = 100:1, Fig. 3B), IL-12 restored cytolytic activity to levels seen at higher doses of APCs. IL-12 was also effective when the blood donors had weak baseline antiinfluenza CTL responses (Fig. 3B). The enhancing effects of IL-12 were also evident with highly purified CD8+ T cells that were stimulated with influenza-infected dendritic cells (Fig. 4). We confirmed that the responding cells in the preparation were primarily CD8+, by staining with monoclonal antibodies at the end of the 7-d culture period (data not shown).

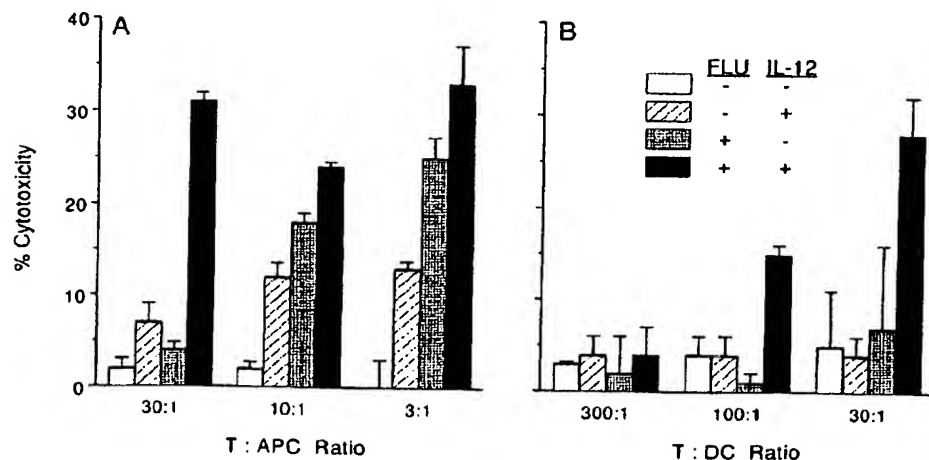


Figure 3. IL-12 enhances anti-influenza virus CTL responses induced by dendritic cells. Partially enriched dendritic cells or "APCs" (A), or purified dendritic cells (B), were uninfected or infected with influenza virus and added at various ratios to bulk T cells. IL-12 was added at 100 pg/ml. Lytic activity was determined after 7 d on syngeneic macrophage-infected targets (E:T ratio = 40:1). Lysis of uninfected targets was < 5% (data not shown).

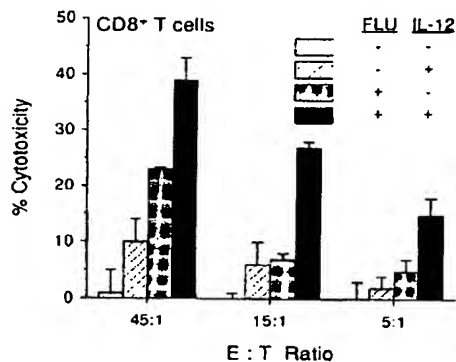


Figure 4. IL-12 enhances cytolytic T cell responses from purified CD8+ T cells. CD8+ T cells were cultured with uninfected or influenza virus-infected APCs (partially enriched dendritic cells) at T:APC ratio of 50:1 in the presence or absence of IL-12 (100 pg/ml).

In summary, IL-12 appears to be most effective in enhancing CD8+ CTL responses when APC numbers are limiting or the CTL response of the donor is weak to begin with.

IFN γ is not responsible for the IL-12-induced enhancement of CTL responses. IL-12 has a major role in facilitating the production of IFN γ by peripheral blood lymphocytes (reviewed in [1]). IL-12 enhanced both antiviral cytolytic responses and IFN γ production in a dose-dependent manner (Fig. 5 A). The highest responses were observed at doses of 100–300 pg/ml, similar to that seen in the T cell proliferation assays (Fig. 2). Similar results were obtained when (a) CD8+ T cells were used (data not shown), or (b) heat inactivated,

After 7 d, effector cells were harvested and lytic activity was determined on syngeneic macrophage-infected targets at various E:T ratios. Lysis of uninfected targets was < 5% at all E:T ratios used (data not shown). This result is typical of experiments with donors exhibiting CD8+ CTL responses of 20–30% without IL-12.

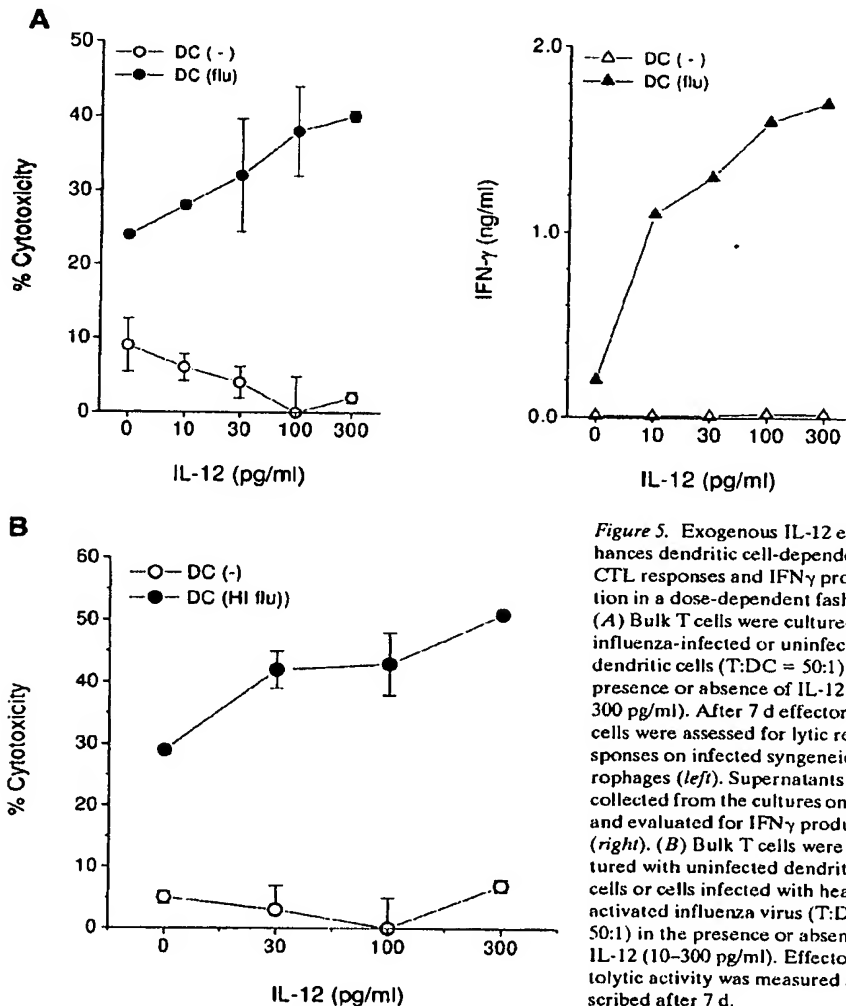


Figure 5. Exogenous IL-12 enhances dendritic cell-dependent CTL responses and IFN γ production in a dose-dependent fashion. (A) Bulk T cells were cultured with influenza-infected or uninfected dendritic cells (T:DC = 50:1) in the presence or absence of IL-12 (10–300 pg/ml). After 7 d effector T cells were assessed for lytic responses on infected syngeneic macrophages (left). Supernatants were collected from the cultures on day 3 and evaluated for IFN γ production (right). (B) Bulk T cells were cultured with uninfected dendritic cells or cells infected with heat inactivated influenza virus (T:DC = 50:1) in the presence or absence of IL-12 (10–300 pg/ml). Effector cytolytic activity was measured as described after 7 d.

Table I. Anti-IFN γ does not Block the CTL-enhancing Function of IL-12

HI-FLU	Treatment	Percentage specific lysis of targets	
		MO [-]	MO [+]
-	goat Ig	0	5
-	anti-IFN γ	0	8
-	IL-12	0	5
-	IL-12 + goat Ig	0	5
-	IL-12 + anti-IFN γ	0	3
+	goat Ig	0	17
+	anti-IFN γ	0	17
+	IL-12	2	36
+	IL-12 + goat Ig	3	45
+	IL-12 + anti-IFN γ	3	34

Highly purified CD8 $^{+}$ T cells were cultured with uninfected dendritic cells or dendritic cells infected with heat-activated influenza virus (HI-FLU) in the presence or absence of interleukin 12 (300 pg/ml). The T:APC ratio was 50:1. Neutralizing goat anti-IFN γ or control goat IgG were added at a final concentration of 25 μ g/ml on the first and fourth days of culture. Antiviral cytolytic activity was assessed on syngeneic uninfected, MO [-], or virus-infected macrophage targets, MO [+], after 7 days. The data are representative of three experiments.

replication deficient virus was used (Fig. 5 B). Even at low doses (10 pg/ml), there was greater than fivefold increase in the quantity of IFN γ produced. This sensitivity to IL-12 has been described in other culture systems (1, 5, 6).

We considered the possibility that IFN γ production induced by IL-12 was mediating the induction of CD8 $^{+}$ CTLs by virus-infected dendritic cells. Endogenous IL-12 production is critical to the generation of NK cell and T cell responses through the production of IFN γ , best demonstrated in models of bacterial and parasitic infections (reviewed in [1]). We added large doses of neutralizing anti-IFN γ antibody to cocultures of T cells and virus-infected dendritic cells in the presence of IL-12. As seen in Table I, the antibody did not block the induction of cytolytic activity in highly purified CD8 $^{+}$ T cell populations. We confirmed that significant amounts of IFN γ were being synthesized in the culture medium in response to IL-12 (up to 2.7 ng/ml). Since our cultures were extensively depleted of NK cells, it is unlikely that they were the source of IFN γ production (see below). We verified that the major responding cells in the cultures after 7 d were CD8 $^{+}$ T cells and not NK cells by FACS $^{\circ}$ analysis.

We determined that IL-12 was responsible for the majority of IFN γ production by CD8 $^{+}$ CTLs. Anti-IL-12 neutralizing antibody blocked the induction of IFN γ release induced by IL-12, as well as the enhanced CTL responses. Anti-IL-2 R α only partially reduced the IFN γ release and CTL responses induced by IL-12 (by \sim 40%, data not shown), indicating that the majority of IFN γ produced is due to IL-12.

Comparison of a panel of cytokines on the enhancement of antiviral CTL responses. IL-12 was compared with a panel of cytokines that are also known to promote CD8 $^{+}$ CTL responses. IL-12 was the most potent enhancer of antigen-dependent CTLs (Fig. 6. A and B). IL-7, a growth factor implicated in the development of CD8 $^{+}$ CTLs (21), appeared to be considerably less effective even when added at 1 ng/ml. Interestingly, IL-4, a key cytokine for the induction of Th2 re-

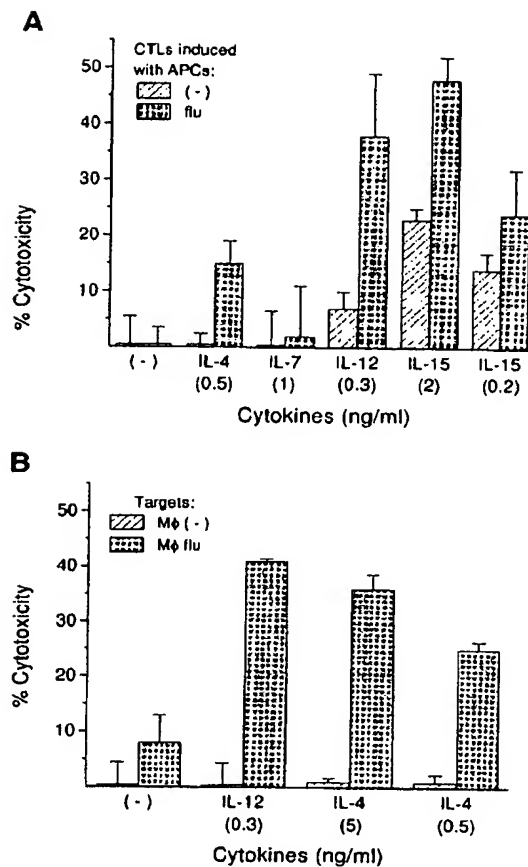


Figure 6. Both IL-12 and IL-4 can selectively enhance virus-specific CTL responses to influenza virus-infected dendritic cells. (A) Bulk T cells were stimulated with uninfected or influenza infected, partially purified dendritic cells (T:APC ratio of 5:1) for 7 d in the presence of various cytokines. CTL responses were tested on syngeneic influenza-infected macrophages. Note the high background with IL-15. (B) Bulk T cells were stimulated with purified dendritic cells at a T:DC ratio of 60:1. After 7 d effector cells were tested for virus specific CTL activity. Lysis of uninfected targets was < 5% at all E:T ratios used (data not shown).

sponses, and which normally counteracts the effects of IL-12, also enhanced the CTL responses significantly. IL-15 increased the CTL response as well, but also caused a substantial rise in the background (Fig. 6, hatched bars), as noted previously in the T cell proliferation assays (Fig. 2). IL-2 was similar to IL-15 in inducing backgrounds and combinations of IL-2 and IL-12 were not synergistic in enhancing CTL responses (data not shown).

Discussion

Activation of antiviral CD8 $^{+}$ CTLs by dendritic cells is enhanced by IL-12. Dendritic cells are specialized antigen-presenting cells for the induction of T cell-mediated immune responses (reviewed in [22]). Their efficacy is based on their ability to prime CD4 $^{+}$ and CD8 $^{+}$ T cells in small numbers and high ex-

pression of adhesins, costimulators, and intracellular vesicles critical for antigen presentation. Another means by which dendritic cells induce potent T cell responses is via the release of IL-12. Using murine TCR transgenic CD4+ T cells, Macatonia et al. (15) showed that dendritic cells induce the differentiation of naive T cells into IFN γ producing Th1 cells by synthesizing IL-12.

Dendritic cells induce potent human antiviral CD8+ CTL responses without the requirement for CD4+ T cells or exogenous cytokines (13). We hypothesized, therefore, that dendritic cells produce IL-12 when stimulating strong antiviral CD8+ CTL responses. However, dendritic cells did not produce significant levels of IL-12 after influenza virus infection or when stimulating either bulk or purified CD8+ T cells. Several stimuli known to produce IL-12 from human macrophages, e.g., LPS, *Staphylococcal aureus* Cowan strain 1, allogeneic T cells, in our hands induced little to no IL-12 when added to human blood dendritic cells. IL-12 may only be mobilized by dendritic cells after certain conditions of T cell activation (15). For example, interaction between CD40L on activated T cells and CD40 on APCs is critical for the priming of Th1 cells (23, 24). The CD8+ CTLs generated in our system may express only low levels of CD40L. Alternatively, the production of IL-12 may critically depend on the source and/or the maturational state of dendritic cells.

The addition of low doses of IL-12 to purified bulk or CD8+ T cells and influenza virus-infected dendritic cells increased both the proliferative and CTL activities substantially (2–20-fold). Our study provides the first evidence of the enhancing effects of IL-12 on human antiviral CD8+ T cell responses. In those instances where donors had weak or poor baseline antiviral responses, the effect of adding IL-12 was dramatic. A strong antiviral immune response was elicited where little to none could be previously detected (Figs. 3 and 6). We feel this is an amplification of a low level of CD8+ T cell memory rather than a primary response, since one would not expect to detect levels of 30–40% CTL lysis in a primary response. While enhancement to such levels are seen with IL-12 in CTL responses to alloantigens or after stimulation with anti-CD3, there the number of responsive T cells is very high (9, 10, 12, 25). IL-12 was also effective when the dose of dendritic cells was less than optimal or limiting (Fig. 3).

IL-12 directly amplifies the antiviral effects of highly purified CD8+ T cells. The amplification of both the proliferative and the antiviral CTL responses correlated with the amount of IFN γ produced in the cultures. In these respects, IL-12 effects on enhancing antiviral CTLs is like the effects on bulk NK cells (5, 6), as long as antigen is being presented by dendritic cells.

Mechanism of IL-12 in enhancing CTL responses. IL-12 likely increases CTL responses through several ways. By functioning as a growth factor (7), it increases the number of T cells responding to antigen (up to twofold as shown here and reported in reference 12), and enhances the production of IL-2 (19, 26) and/or the expression of the IL-2 high affinity receptor (CD25, [27]). The effect of IL-12 may be direct since it is not blocked by anti-IL-2R α (7, 10–12). Activation of NK cells by IL-12 is similarly not dependent on cytokines such as IL-2 (1, 5, 6). In our system IL-2 was critical, since the addition of anti-IL-2R α antibody substantially blocked CTL responses as described by others (9).

IL-12 may also upregulate the expression of perforin (11), serine esterase levels and cytotoxic granules in CD8+ CTLs (10, 12). A direct increase in the the CTL precursor frequency

has also been postulated. However, we were unable to measure enhanced CTLp levels under limiting dilution conditions (data not shown).

IL-12-induced IFN γ could directly enhance antiviral responses through its effects on infected target cells. This is one mechanism by which NK cells clear virus (28). We found, however, that the addition of neutralizing anti-IFN γ antibodies did not block either the induction of or killing capacity of CD8+ CTLs in the presence of IL-12. These findings are in concordance with those of Mehroratra et al. (12) who showed that the synergistic effects of IL-2 and IL-12 on CTL generation were not blocked by anti-IFN γ , and Bloom et al. (11) in the murine system where allospecific CTL generation was studied.

Our results do not rule out a role for IFN γ in antiinfluenza responses, however, or of cytokines such as TNF α which are also produced in response to IL-12. This is because our assays are designed to measure cytolytic activity of virus-infected target cells vs elimination of virus through other inhibitory mechanisms. IFN γ can clear infection by affecting viral RNA synthesis and stability, replication, and induction of mediators such as nitric oxide (29). Such mechanisms, rather than direct cytolytic activity, are thought to be the primary means by which hepatitis virus and other cytopathic infections are cleared (30).

When compared to other T cell growth factors, IL-12 appeared to be the most efficient at enhancing CTL responses. IL-2 and IL-15, while partly effective, gave high background responses possibly because of effects on IL-2 R β expressing T cells. IL-15 was tested because it can amplify dendritic cell-dependent T cell responses (31) and increases antigen-specific proliferative T cell responses (19). IL-12 and IL-15 are synergistic in augmenting NK cell CTL function and IFN γ production (18), but were not evaluated together because of the observed background activity of IL-15. Besides inducing the development of Th2 cells from naive CD4+ T cells, IL-4 can augment CTL responses including antiinfluenza CTL responses (32), as shown here (Fig. 6). The pathway by which IL-4 enhances dendritic cell-dependent CTL responses is not known. However, IL-4 is known to prime PBMC for IL-12 production in response to LPS or *S. aureus* (33). It is possible that the effects of IL-4 seen here are mediated through dendritic cells that have been primed for IL-12 production. IL-7, which has been used with dendritic cells to induce CTL responses (21) was not effective in our studies.

Restricted requirements for IL-12 in antiviral responses. The regulatory influence of IL-12 on cellular immune responses is most striking in animal models of bacterial and protozoal infection (reviewed in references 1 and 2). Surprisingly, less is known about the role of IL-12 in T cell-mediated resistance to viral infections. For instance, little is known about whether IL-12 plays a role in the development of antiviral CD8+ CTLs.

In two animal models of virus infection, lactate dehydrogenase elevating virus (34) and MCM virus infections (28), either message for IL-12 or protein is detected in macrophages and splenic cells shortly after infection. However, the primary mode of virus clearance, at least in MCMV infection, appears to be via NK cells through secretion of IFN γ , rather than CD8+ T cells. This antiviral pathway is blocked with anti-IL-12 antibody or enhanced with administration of low dose IL-12 (28). In contrast, CD8+ T cell expansion is unaffected by IL-12 neutralizing antibody. In other virus infections IL-12 is not readily detectable, e.g., LCMV infection, and viral clear-

ance is primarily dependent on the activation of CD8+ CTLs. While low doses of IL-12 enhance splenic CD8+ T cell numbers in LCMV-infected mice and decrease viral replication, cytolytic activity is not increased (35). Furthermore, the administration of anti-IL-12 antibody has no demonstrable effect on late T cell responses (28). Thus we would predict that the ability to rapidly induce significant IL-12 levels in vivo will determine whether NK cells are major contributors to antiviral defense.

In humans, there is evidence to suggest that IL-12 has a critical function in the progression of HIV infection (26). PBMCs from patients infected with this virus are deficient in their production of IL-12, and have suboptimal Th1 immune responses (36). Addition of IL-12 to T cells in vitro, restores recall responses to antigens (19, 26). It is presumed, but not established, that IL-12 deficiency also affects antiviral CD8+ T cell development and function.

In the case of influenza virus, significant levels of IL-12 were not produced by either macrophages or dendritic cells after infection in vitro. IL-12 is reportedly produced in the lungs of mice as early as day 3 after infection with influenza virus, but it is not known which cells synthesize the cytokine (37). Neutralizing anti-IL-12 antibodies increase morbidity in virus-infected mice compared with uninjected animals, suggesting that the cytokine is essential for a potent antiviral immune response (28). Influenza virus infection is known to elicit IFN γ production in infected human PBMCs (38). Perhaps CD40L-bearing helper T cells are major inducers of IL-12 production by dendritic cells in infected lungs. Whether IL-12 is a requisite in vivo for clearance of influenza virus in humans cannot be determined from these experiments. Optimal clearing of influenza virus after infection likely requires CD8+ CTL effector activity (39).

Applications. IL-12 may be a useful immunomodulator when used in conjunction with dendritic cells, to enhance antigen-specific class I restricted CD8+ CTL responses in viral infections and tumors. Peptide pulsed dendritic cells induce protective immune responses and also cause tumor regression of established metastases (40–42). The concomitant use of IL-12 may reduce the need for large numbers or doses of dendritic cells. Similarly, by using dendritic cells, it may be feasible to use very low doses of IL-12. Large doses of IL-12 have exhibited toxicities in both humans and in animals (43). In HIV infection, PBMC from infected patients demonstrate impaired IL-12 production (36). Immunotherapy may be most effective in later stages of the disease when CD4+ T cell counts fall and CD8+ CTL function declines. Here, IL-12 could concomitantly enhance virus-specific CTL responses as well as CD4+ helper cell responses, the latter as previously described (26).

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Rapamycin inhibits macropinocytosis and mannose receptor-mediated endocytosis by bone marrow-derived dendritic cells

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Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that use 2 major pathways for antigen uptake: constitutive macropinocytosis and mannose receptor-mediated endocytosis. Efficient endocytosis is critical for DCs to fulfill their sentinel function in immunity. We investigated the influence of the immunosuppressive macrolide rapamycin on macropinocytosis of fluorescein isothiocyanate (FITC)-albumin and mannose receptor-mediated endocytosis of FITC-

dextran by murine bone marrow-derived DCs by flow cytometry. The data show that (1) at a low, physiologically relevant concentration (1 ng/mL), rapamycin impairs macropinocytosis and mannose receptor-mediated endocytosis; (2) the effects are independent of DC maturation and can be demonstrated specifically in immature CD11c⁺ major histocompatibility complex (MHC) class II^{lo} DCs by 3-color flow cytometry; (3) inhibition of endocytosis is not related to apoptotic cell death;

and (4) molar excess of the structurally related molecule FK506 inhibits the actions of rapamycin. The inhibitory effects of rapamycin on DC endocytosis were confirmed in vivo. To our knowledge, this is the first report that a clinically relevant immunosuppressant inhibits DC endocytosis. (Blood. 2002;100:1084-1087)

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Introduction

Dendritic cells (DCs) arise from CD34⁺ bone marrow (BM) stem cells and represent a heterogeneous population of ubiquitously distributed antigen-presenting cells (APCs) that play critical roles as initiators and modulators of immune responses.^{1,2} Among the most striking features underlying the efficiency of DCs as APCs is their unsurpassed capacity to take up antigens via constitutive macropinocytosis and mannose receptor-mediated endocytosis³ and to subsequently process and present major histocompatibility complex (MHC)-antigen complexes on their surface.¹ The capacity of DCs to endocytose and to present antigens is under tight developmental control: immature DCs are excellent at internalizing antigens but express low surface levels of MHC class II molecules, whereas mature DCs down-regulate endocytotic activity and up-regulate MHC class II and costimulatory molecules (CD40, CD80, CD86) that promote T-cell activation.¹ Anti-inflammatory drugs such as corticosteroids⁴ or salicylates⁵ suppress DC maturation and as a consequence enhance their endocytotic activity. Recent reports point toward Rho family proteins Cdc42⁶ and Rac,⁷ as well as aquaporins,⁸ as important factors that regulate DC endocytosis. Rapamycin is a potent immunosuppressive macrolide, isolated from *Streptomyces hygroscopicus*, that inhibits downstream signaling from the targets of rapamycin proteins (TORs) by forming a complex with its intracellular receptor FK506-binding protein 12 (FKBP12) and TORs.⁹ It is used clinically to prevent and treat allograft rejection.¹⁰⁻¹² Interaction of the rapamycin-FKBP12 complex with TORs results in inhibition of multiple biochemical pathways (eg, p70 S6 kinase, cyclin-dependent kinases, transla-

tional effector proteins) that are critical for cytokine/growth factor-induced cellular proliferation, ribosome biosynthesis, translation initiation, and cell cycle progression into S phase.^{9,13} In view of the paucity of information concerning the influence of rapamycin on APC function and its well-documented inhibitory effects on protein synthesis, we analyzed the impact of rapamycin on DC endocytosis. Our results indicate that rapamycin is an inhibitor of DC endocytosis in vitro and in vivo and that molar excess of the structurally related immunophilin ligand FK506 partially reverses its inhibitory effects.

Study design

Generation of BM-derived DCs

Male C57BL/10J (1-A^b) mice, 8 to 12 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME). BM-derived DCs were generated and characterized as described,⁵ with minor modifications. Briefly, BM cells were cultured for 7 days in RPMI-1640 with 10% heat-inactivated fetal calf serum (FCS), L-glutamine, nonessential amino acids, sodium pyruvate, penicillin-streptomycin, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2-mercaptoethanol (2-ME) (all from Life Technologies, Gaithersburg, MD), 1000 U/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Kenilworth, NJ) ± 1000 U/mL murine interleukin 4 (IL-4) (R&D Systems, Minneapolis, MN). At day 2, 1-100 ng/mL rapamycin (Sigma, St Louis, MO) ± 20-250 ng/mL FK506 (Prograf for intravenous use, Fujisawa Healthcare, Deerfield, IL) was added. Every 2 days, 75% supernatant was

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replaced with fresh cytokine-containing medium (\pm rapamycin, FK506). On day 4, nonadherent cells were removed; on day 7, $\geq 50\%$ of the nonadherent cells expressed CD11c.

In vivo administration of rapamycin

Rapamycin (Wyeth-Ayerst, Princeton, NJ) was dissolved in 51% wt/vol polyethylene glycol 300 (PEG300), 2.5% wt/vol polysorbate 80, 10% vol/vol ethanol (vehicle; reagents from Sigma). Chinese hamster ovary (CHO) cell-derived rhuman Flt3 ligand ($10\mu\text{g/kg/d}$, intraperitoneally; Immunex, Seattle, WA) was used to expand DCs in mice over a period of 10 days.¹⁴ Animals were injected with rapamycin (0.5 mg/kg/d , intraperitoneally) or vehicle at the same times. Spleens were disaggregated with fine scissors and passed through a nylon cell strainer (Falcon Becton Dickinson, NJ) to obtain a single-cell suspension. Erythrocytes were lysed with 0.83% wt/vol NH_4Cl .

Endocytosis

Quantitative analysis of endocytosis was performed as described,⁵ with minor modifications. Cells (5×10^5) were incubated with $5\mu\text{g/mL}$ FITC-albumin (Sigma) or 0.1 mg/mL FITC-dextran (MW 42000, Sigma) at either 37°C or 4°C for 60 minutes (for in vivo-expanded DCs, $500\mu\text{g/mL}$ FITC-albumin and 1 mg/mL FITC-dextran for 40 minutes). Endocytosis was stopped by 2 washes in ice-cold 0.1% sodium azide/ 1% FCS/phosphate-buffered saline (PBS). Cells were stained for CD11c (HL3) and, in some experiments, for MHC class II expression (IA β β -chain, 25-9-17) as described (monoclonal antibodies from BD PharMingen, San Diego, CA).⁵

Apoptotic cell death

Apoptosis was analyzed by staining of phosphatidylserine translocation with FITC-annexin-V in combination with the vital dye 7-AAD (BD PharMingen) and detection of DNA fragmentation by terminal deoxynucleotidyl transferase-mediated fluorescein-2'-deoxyuridine 5'-triphosphate (dUTP) labeling (TUNEL assay; Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Positive controls included deoxyribonuclease (DNase) treatment and ultraviolet C (UVC) irradiation (60 J/m^2) in the TUNEL and annexin-V assay, respectively. Cells were costained for CD11c expression and analyzed by means of an EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL).

Statistics

Statistical analysis was performed with a 2-tailed Student *t* test. Normal distribution of values was proved by the Kolmogorov-Smirnov test.

Results and discussion

Analysis of GM-CSF+IL-4-expanded, BM-derived DCs harvested at day 7 of culture revealed a reduced capacity of cells exposed to rapamycin to exhibit macropinocytosis of FITC-albumin and mannose receptor-mediated endocytosis of FITC-dextran. This was evident with respect to both the incidence (Figure 1A, B) and the mean fluorescence intensity (MFI; Figure 1D) of CD11c $^+$ cells. CD11c is a very reliable marker for murine DCs¹⁵ and is not expressed in significant amounts by murine macrophages.^{5,16} Because DC maturation is associated with marked down-regulation of endocytotic capacity,³ we questioned whether these effects might be related to a stimulatory effect of rapamycin on DC maturation. However, rapamycin-exposed CD11c $^+$ DCs displayed significantly decreased surface MHC class II (Figure 1C) and costimulatory molecules (data not shown), indicating that they were more immature than untreated, control cells. It could be argued that the analysis of a fixed number of DCs by fluorescence-activated cell sorting (FACS) might mask an inhibitory effect of rapamycin on the differentiation of precursor cells into DCs. As

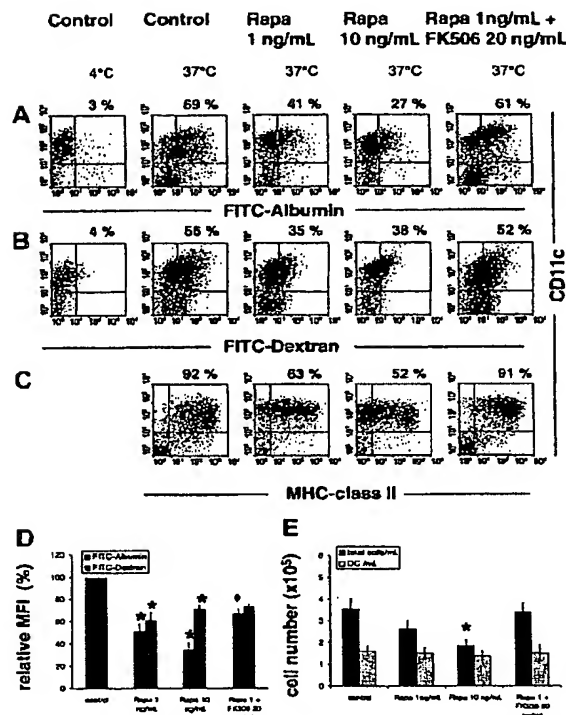


Figure 1. Rapamycin inhibits endocytosis by GM-CSF+IL-4-expanded DC. (A-D) BM-derived DCs were expanded for 7 days as described in "Study design"; rapamycin (Rapa) was added at day 2 (\pm FK506) at the concentrations indicated. FITC-albumin and FITC-dextran internalization at 37°C and 4°C (negative control), MHC class II (IA β β -chain), and CD11c expression were analyzed by flow cytometry. In all experiments, CD11c $^+$ cells were analyzed to determine endocytosis and MHC class II expression specifically in DCs. (A-C) Numbers indicate the percentage of CD11c $^+$ cells that were positive for the marker indicated. (D) Data represent mean values (\pm SE) of CD11c $^+$ cells after subtraction of background fluorescence (4°C). (E) Comparison of total cell and DC numbers on day 7 of culture. (D, E) Differences between paired cultures were compared by 2-tailed Student *t* test for paired samples ($*P < .05$ vs control; $\diamond P < .05$ vs Rapa 1). (A-D) Results are representative of 4 (FITC-dextran) and 6 (FITC-albumin) separate experiments. (E) Results are representative of 6 separate experiments.

shown in Figure 1E, however, rapamycin inhibited *total cell expansion* in a dose-dependent manner (antagonizable by a molar excess of FK506) but did not significantly block the *differentiation* of precursor cells into CD11c $^+$ DCs, as evidenced by the comparable yield of CD11c $^+$ DCs. Achievement of similar DC yields was due to consistently increased percentages of CD11c $^+$ DCs with characteristic DC morphology in the rapamycin-treated cultures.

Because additional experiments indicated that the inhibitory effect of rapamycin on DC maturation was IL-4 dependent (H.H., T.T., unpublished observations, October 2001), we expanded DCs with GM-CSF only. Generation of murine DCs in GM-CSF is a well-established culture method.¹⁷ Additionally, to determine more precisely the endocytotic activity of homogenous DCs at the same stage of maturation, we specifically analyzed immature MHC II $^{\text{lo}}$ DCs. These experiments confirmed the inhibitory effects of rapamycin on DC endocytosis and indicated that they were not IL-4 related (Figure 2A-C). Again, low concentrations of 1 ng/mL rapamycin (1.1 nmol) were sufficient to significantly and markedly suppress endocytotic activity. When 1 ng/mL rapamycin was used, the relative MFI of immature MHC class II $^{\text{lo}}$ CD11c $^+$ DCs compared

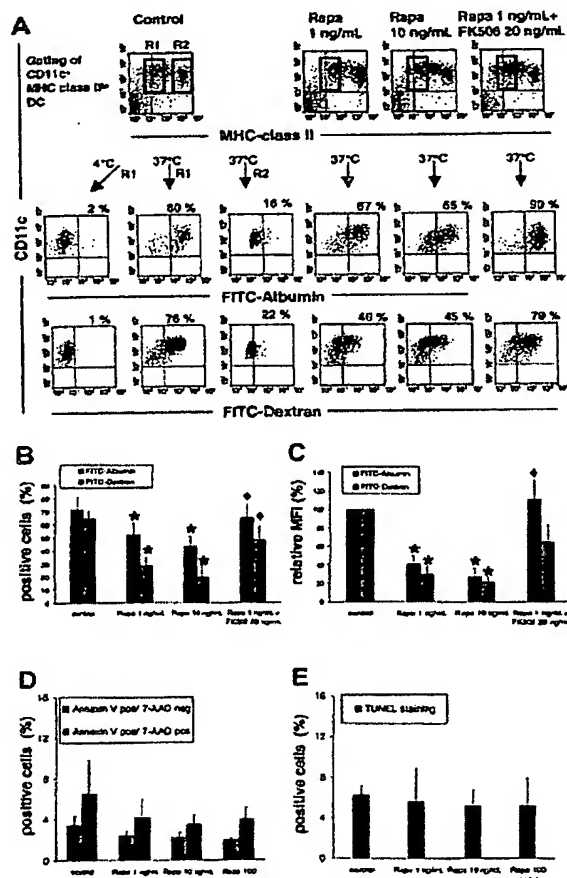


Figure 2. Rapamycin inhibits endocytosis by GM-CSF-expanded immature BM-derived DCs. (A-E) BM-derived DCs were expanded for 7 days with GM-CSF only, as described in "Study design"; rapamycin (Rapa) was added at day 2 ($1 \mu\text{M}$ FK506) at the concentrations indicated. (A-C) FITC-albumin and FITC-dextran internalization at 37°C and 4°C (negative control), MHC class II (IA β β -chain), and CD11c expression were analyzed by 3-color flow cytometry. CD11c $^+$ MHC class II $^+$ cells were gated to determine endocytosis specifically in immature DCs. Regions R1 and R2 show endocytotic activity of immature MHC class II $^+$ and mature MHC class II $^+$ DCs, respectively. (A, B) Numbers indicate the percentage of CD11c $^+$ MHC class II $^+$ cells positive for the marker indicated. (B, C) Data represent mean values (\pm SE) of CD11c $^+$ MHC class II $^+$ cells after subtraction of background fluorescence (4°C). Differences between paired cultures were compared by the 2-tailed Student *t* test for paired samples ($*P \leq .01$ vs control; $\dagger P < .05$ vs Rapa 1). (D, E) Apoptosis of CD11c $^+$ DC was determined by annexin-V/7-AAD staining (D) and the TUNEL assay (E). Numbers represent mean values (\pm SE). Incidences of apoptotic (annexin-V positive/7-AAD negative or TUNEL positive) or secondary necrotic (annexin-V positive/7-AAD positive) cells were consistently lower than 10%. There was a trend toward decreased apoptosis/cell death in rapamycin-treated cultures, but the differences were not statistically significant. (A-C) Results are representative of 4 separate experiments. (D, E) Results are representative of 2 (TUNEL assay) and 3 (annexin-V/7-AAD assay) separate experiments.

with controls was $< 42\%$ and $< 32\%$ with respect to FITC-albumin and FITC-dextran, respectively (Figure 2C). Given that mean trough whole blood levels of rapamycin in patients are 17.3 ng/mL (5 mg rapamycin/d)¹⁸ and that the free plasma fraction is 8%, these concentrations are clinically highly relevant. When rapamycin was added at day 6 of culture, it still inhibited DC endocytosis, but the overall effect was weaker (relative MFI 69%

and 53% for FITC-albumin and FITC-dextran, respectively, at 5 ng/mL rapamycin).

Binding of rapamycin to FKBP12 and TOR inhibition can be antagonized in vitro by the structurally related immunophilin ligand FK506.^{19,20} Whereas FK506 alone (20 ng/mL) did not interfere with DC endocytosis (data not shown), addition of 20 ng/mL FK506 to 1 ng/mL rapamycin (22.3-fold molar excess) antagonized the inhibitory effects of rapamycin, indicating that these were related to FKBP12-mediated TOR inhibition (Figures 1A, B; 2A-C). However, the antagonistic effect of FK506 was incomplete, especially with respect to mannose receptor-mediated endocytosis. Similar results were obtained over a wide range of drug concentrations (1–20 ng/mL rapamycin, 10–250 ng/mL FK506) and at different FK506/rapamycin ratios (10–55 molar). This suggested that other FKBP might also be involved in endocytosis inhibition. One candidate is FKBP25, which has > 100 times greater binding affinity for rapamycin than FK506.²¹

Having established that rapamycin inhibited DC endocytosis, we analyzed whether this effect was due to increased apoptotic cell death. In contrast to a recent report,²² the incidence of apoptosis at day 7 of culture was consistently low ($< 10\%$) and was not affected significantly by rapamycin, even at a suprapharmacological dose of 100 ng/mL, as determined independently by annexin-V/7-AAD and TUNEL staining (Figure 2D, E). Similar results were obtained with GM-CSF+IL-4-expanded DCs and at day 4 of culture (data not shown). To investigate the in vivo relevance of DC endocytosis inhibition, we analyzed endocytotic activity in splenic DCs of animals that were injected with rapamycin (0.5 mg/kg/d for 10 days, ip) or vehicle and in which DCs were expanded with Flt3 ligand as we have described.¹⁴ After 10 days, the animals were killed and FITC-albumin and FITC-dextran uptake by freshly isolated splenic CD11c $^+$ DCs was analyzed. The phenotype of

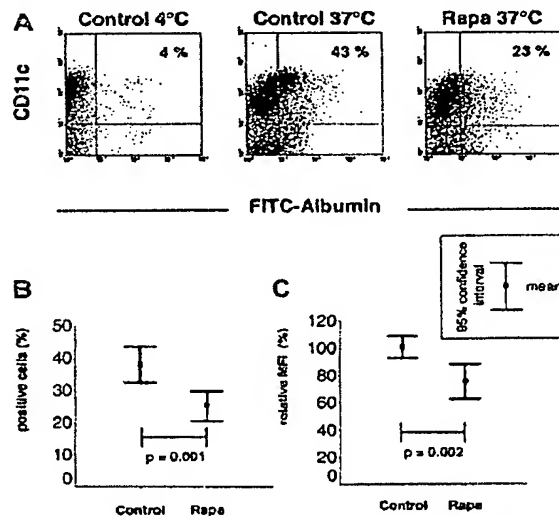


Figure 3. In vivo administration of rapamycin inhibits endocytosis by Flt3 ligand-expanded splenic DCs. (A-C) DCs were expanded in vivo with Flt-3 ligand and animals were injected with either rapamycin or vehicle, as described in "Study design." Endocytotic activity was measured by analyzing FITC-albumin uptake at 37°C and 4°C (negative control) and relative MFI in comparison with animals injected with vehicle only. Results are representative of 8 animals per group and were obtained in 5 independent experiments. Differences between the groups were compared by the 2-tailed Student *t* test for independent samples. Similar findings were obtained with respect to FITC-dextran uptake, as described in "Results and discussion."

these DCs was immature in both treatment groups. As shown in Figure 3, CD11c⁺ DC of rapamycin-treated animals displayed significantly reduced macropinocytotic activity, with respect to both the number of positive cells and the relative MFI ($P = .001$ and $P = .002$, respectively). Similar findings were obtained with respect to FITC-dextran uptake (41.5% positive cells vs 24.8% in rapamycin-injected animals, $P < .05$; relative MFI 74.7%, $P < .05$).

The results of this study suggest that rapamycin can interfere with immune responses at a very early stage by inhibiting DC endocytosis. Thus, rapamycin targets a unique function of DCs that influences the induction of immunity against microbial pathogens (eg, *Salmonella*²³) and allergens.²⁴ This effect may also suppress indirect alloantigen processing following transplantation.

The precise mechanisms by which rapamycin inhibits DC endocytosis remain to be determined. In this context, it is important to note that the Rho GTPases CDC42 and Rac that interfere with

the endocytotic activity of DCs^{7,8} complex with and activate the p70 S6 kinase²⁵ that belongs to the central signaling pathway disrupted by rapamycin.⁹ In addition, rapamycin's inhibition of TOR signaling down-regulates protein translation and has been demonstrated to suppress actin synthesis.²⁶ Because, to our knowledge, no clinical immunosuppressant has been reported to inhibit DC endocytosis, these findings may provide further incentive for trials of rapamycin in clinical settings other than transplantation, for example, in autoimmune disease.

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IL-12 increases CD80 expression and the stimulatory capacity of bone marrow-derived dendritic cells

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Abstract

Dendritic cells (DC) are potent antigen-presenting cells derived from CD34 bone marrow stem cells. They undergo a series of maturational steps that allow them to stimulate primary T cell responses. Several cytokines are known to contribute to this process. In this study murine DC maturing from bone marrow progenitors under the influence of granulocyte macrophage colony stimulating factor and tumour necrosis factor- α were found to produce IL-12 as measured by ELISA and by flow cytometry to detect intracellular cytokine. Administration of additional IL-12 from day 3 to 7 of culture altered the function and phenotype of DC; enhanced stimulation of T cell proliferation by DC in allogeneic mixed leukocyte reactions was associated with an increase in the surface expression of CD80 on DC. These effects were dose dependent, and were consistently seen with IL-12 at 25 ng/ml and were less marked with IL-12 at 50 ng/ml. These results show that IL-12 is both produced by DC and can increase their stimulatory capacity. The findings suggest that there may be an autocrine effect of IL-12 on DC maturation and function.

Introduction

Bone marrow-derived dendritic cells (DC) are specialized to acquire and process antigens in peripheral tissues. They transport antigen via the afferent lymphatics to lymph nodes, and mature and up-regulate histocompatibility antigens and co-stimulatory molecules such as CD40, CD80 and CD86. In the lymph nodes, DC have less capacity to process antigen but possess the unique ability to activate naive T cells. DC are also efficient stimulators of secondary T cell immune responses (1).

IL-12 is a heterodimeric cytokine composed of covalently linked p35 and p40 subunits encoded on two separate genes (2). The p35 mRNA is constitutively expressed by a variety of cell types, whereas p40 expression is correlated with the production of bioactive p70 by antigen-presenting cells (3). IL-12 is secreted in three main forms, i.e. the p70 heterodimer, p40 homodimers and p40 monomers. The function of the p40 moieties is not yet established *in vivo*. However, p70 enhances the proliferation and cytolytic activity of NK cells and T cells, and stimulates IFN- γ production by these cells. DC produce IL-12 which promotes the development of CD4 T_H1 cells from naive T cells (4–6). CD40 ligation on DC enhances both their

maturation and stimulatory capacity in the mixed lymphocyte reaction (MLR) and induces the production of IL-12 in these cells (7–9). Since cytokines can amplify the antigen-presenting function of DC, it is possible that IL-12 could play a direct role in this process.

The aims of this study were (i) to see if bone marrow-derived DC produce IL-12 at different time points during maturation, and (ii) whether IL-12 administration during DC maturation altered cell numbers, phenotype and function.

Materials

Mice

Specific pathogen-free female BALB/c and CBA mice, 6–10 weeks old, were obtained from Northwick Park Institute for Medical Research.

Media and reagents for cell culture

The culture medium (CM) was RPMI 1640 (Dutch modification; Sigma, Poole, UK) supplemented with 10% FCS (Gibco, Paisley, UK), 100 U Penicillin (Gibco), 100 μ g/ml streptomycin

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(Gibco), 2 mM glutamine (ICN Flow, Irvine, UK), 2 mercapto-ethanol 10^{-5} M (Sigma). Wash medium was identical to the CM but contained bicarbonate-buffered RPMI 1640 instead of the Dutch modification. Granulocyte macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor (TNF)- α (R & D Systems, Oxford, UK) were used at concentrations of 100 and 50 U/ml respectively. IL-12 was a generous gift from Dr M. Gately (Roche Discovery, Nutley, NJ) and was used at 10–50 ng/ml.

DC

DC were derived from bone marrow precursors as previously described (10). Briefly, bone marrow was obtained from the femura and tibiae of BALB/c mice. Mononuclear cells were isolated by centrifuging on Lympholyte M (Cedarlane, Hornby, Ontario, Canada). The interface cells were collected, washed and suspended in CM at a concentration of 10^6 cells/ml, and GM-CSF and TNF- α were added. After a 2–4 day incubation the non-adherent cells were centrifuged at 600 *g* over metrizamide (Nyegaard, Oslo, Norway; 13.7% w/v) to collect the low-density cell population (LDC) in which DC are found. The LDC were resuspended in CM at a cell concentration of 5×10^5 cells/ml supplemented with GM-CSF and TNF- α . IL-12 at a concentration from 10–50 ng/ml was then added to the cultures. The medium was changed every 2–3 days and IL-12 together with the other two cytokines were replenished over the next 5 days. After a total of 10–14 days of culture the non-adherent cell population was again centrifuged over a metrizamide gradient, and the LDC washed and suspended in CM. DC numbers were estimated by light microscopy and Trypan blue was used to estimate cell viability. Normal cell viability was 95–100%.

Flow cytometry

The DC were labelled with directly conjugated FITC mAb: mouse I-A^d (clone AMS 32.1, IgG2b), mouse H-2D^d (clone 34-2-12, IgG2a), hamster anti-mouse CD11c (clone HL3, hamster IgG), hamster anti-mouse CD54 (clone 3E2, hamster IgG) and rat anti-mouse CD11a (clone C71/16, rat IgG2a). Biotinylated mAb employed were: rat anti-mouse CD40 (clone 3/23, rat IgG2a), rat anti-mouse CD80 (clone 1G10, rat IgG2a), rat anti-mouse CD86 (GL1, IgG2a), rat anti-mouse CD8 (clone ABC, rat IgG2a), hamster anti-mouse CD95 (clone Jo2, hamster IgG) and rat anti-mouse IL-12 (clone C17.15, rat IgG2a). The FITC-conjugated antibodies were purchased from PharMingen (San Diego, CA). The biotinylated IL-12 antibody was obtained from Genzyme (Cambridge, MA) and the other biotin antibodies were obtained from PharMingen. Avidin-FITC and streptavidin-phycoerythrin (PE) (Becton Dickinson, Mountain View, CA) were used to label the biotinylated antibodies.

Twenty percent FCS in FACScan buffer was used to prevent non-specific antibody binding. FITC-conjugated and biotinylated mAb were added to the LDC and the samples were left on ice for 30 min. The cells were then washed in FACScan buffer and avidin-FITC was added. After 20 min these samples were washed and 50 μ l of 1% paraformaldehyde added for overnight fixation. Fluorescence profiles were generated on a FACScan flow cytometer (Becton Dickinson). Histogram and density plots were produced by the CellQuest (version

1.0) software package. Viable DC were selected after gating on forward and side scatter with dead cells excluded by propidium iodide staining. The cells which were selected in the gate set around the DC cluster expressed high levels of MHC class II, and were stained by murine markers for DC such as NLDC-145, 33D1 and CD11c as previously described (10). Several antigens were not detected including CD19 (B cells), CD3 ϵ (T cells) and CD115 (macrophages). Ninety percent of the selected cells expressed the murine-specific DC marker NLDC-145. The user parameters set for experiments involving control and IL-12-treated DC were as follows: FSC linear 5.67, SSC log 242 and FL1 log 460.

Intracellular flow cytometry

Monensin at a concentration of 1.5 mM was added to 5×10^5 LDC to ensure intracellular cytokine retention. The LDC/monensin preparation was incubated for 6 h at 37°C. The cells were then washed twice in FACScan buffer containing 20% FCS. Cytoperm A (Serotec, Oxford, UK) was added, the cells kept at room temperature for 15 minutes for fixing and then washed again in FACScan buffer with 20% FCS. Cells were then exposed to permeabilizing agent, Cytoperm B (Serotec), at room temperature for 15 min. The cells were then double labelled at room temperature for the p40 subunit of IL-12 (rat anti-mouse IgG2a; Genzyme) and murine DC-specific markers NLDC-145 (cell line rat IgG2a) and 33D1 (cell line rat IgG2b). The fluorescent cytokine profiles were generated on the FACScan flow cytometer as described above. The user parameters for these experiments were FSC linear 5.67, SSC log 242, FL1 log 440, FL2 log 475, and compensation FL1–FL2 0.6 and FL2–FL1 29.5.

Lymphocyte proliferation assays

Lymphocyte proliferation assays were performed in 20 μ l hanging drop cultures on inverted Terasaki plates (11). CBA lymph nodes were pressed through a wire gauge in medium, washed once and used as responder cells for the BALB/c-derived bone marrow DC. On day 3 the cultures were pulsed with 1 μ g/ml [³H]thymidine with a sp. act. of 2 Ci/mM (Amersham International, Amersham, UK) for 2 h and then transferred by blotting on to filter discs. The thymidine uptake into proliferating cells was measured in the acid-insoluble fraction on the filter discs in a liquid scintillation counter (Canberra Packard 2000 CA Tri-Carb). This technique gives values that are lower than usual in the 200 μ l culture systems, but they reflect accurately the DNA synthesis occurring in these cultures. Data points for each experiment shown represent the mean c.p.m. of triplicate cultures. ANOVA and Student's *t*-test were used to identify significant effects.

Cytokine measurement

IL-12 p70, IL-12 p40, IFN- γ and IL-4 levels were determined by the Intertest solid-phase ELISA kit (Genzyme). Microtiter plates were coated with specific antibodies to capture the cytokine of interest in cell culture supernatants. A second layer antibody was then added. The detection of bound cytokine was achieved using a horseradish peroxidase-conjugated antibody and a colour substrate. Cytokine concentrations were determined with a standard curve derived from known amounts of the relevant cytokine using absorbance

readings at 450 nm on a spectrophotometer. There was no interference from p40 in the culture supernatants with the p70 heterodimer. The lower limits of detection for IL-12 were 10 pg/ml for p40 and 5 pg/ml for p70 and the other cytokines.

Results

DC derived from bone marrow preparations secrete IL-12

The presence of IL-12 in supernatants from maturing LDC (10^6 cells) was assessed on day 5 and 8 of culture. IL-12 p40 was found in excess of IL-12 p70 as previously described (6). The values on day 5 were 120 pg/ml of IL-12 p40 and 20 pg/ml of IL-12 p70. Three days later the amounts of IL-12 p40 and IL-12 p70 had increased to 150 and 24 pg/ml respectively. Similar values for IL-12 were found on repeat experiments. There were no lymphocytes observed in these cultures on light microscopy, which was confirmed on flow cytometry using the T and B cell markers CD3 ϵ and CD19. IFN- γ and IL-4 and IL-10 were not detected in culture supernatants at these time points.

Flow cytometry was used to detect intracellular cytokine production by DC derived from bone marrow precursors. IL-12 p40 was identified in cells which express DC markers (Fig. 1). A higher proportion of the cells labelled with NLDC than 33D1 as previously described (10). Almost all the cells producing IL-12 were positive for NLDC and most labelled with 33D1. Eighteen percent of the NLDC-labelled cells and 38% of the 33D1 positive cells were positive for IL-12 p40. Non-specific intracellular binding by the p40 antibody was excluded by showing that IL-12 p70 in excess inhibited intracellular p40 labelling and there was no labelling for IL-12 p40 in the absence of monensin (Fig. 1). In addition, an isotype antibody (rat anti-mouse IgG2a) was also used as a negative control for IL-12 p40 (data not shown).

IL-12-treated DC exhibit differences in cell surface phenotype and function

IL-12 administration did not alter the proportion or numbers of DC derived from bone marrow progenitors as assessed by light microscopy and flow cytometry (data not shown); there was no evidence for any toxic effects of IL-12 at the highest dose. The effect of IL-12 administration at 10, 25 and 50 ng/ml on the surface phenotype of DC after 10–14 days of culture was examined by flow cytometry. A most striking finding was that 25 ng/ml of IL-12 up-regulated cell surface expression of CD80 on DC. The geometric mean fluorescence intensity of the control DC was significantly higher following IL-12 treatment with 25 ng/ml, e.g. 39.1 compared with 59.9 (Fig. 2). The influence of 50 ng/ml of IL-12 on CD80 expression on DC was more variable; in some experiments (three out of 10) CD80 expression was also increased (data not shown). There were no reproducible differences in CD80 expression with the lowest dose of IL-12 (10 ng/ml) and mean fluorescence from labelling with a number of other antibodies showed no reproducible differences with any dose of IL-12 (Fig. 2).

DC treated with IL-12 at 25 ng/ml stimulated higher levels of T cell proliferation than untreated cells (Fig. 3). DC treated with 50 ng/ml usually did not increase T cell proliferation in the MLR (seven out of 10 experiments), although in the three

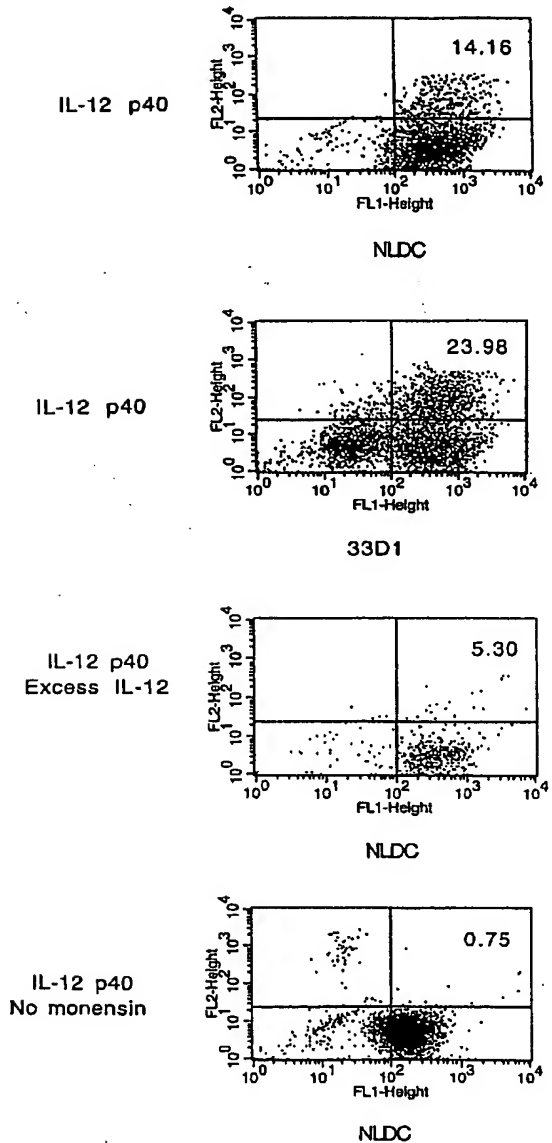


Fig. 1. Flow cytometry to measure intracellular IL-12 in DC was performed using double labelling with a biotinylated IL-12 p40 antibody and NLDC or 33D1 as markers for DC as indicated on the axes. Controls for IL-12 p40 labelling were DC incubated with excess IL-12 p70 and cells prepared in the absence of monensin which both showed little labelling, and the results are presented for the cells labelled with NLDC. The percentage of the total cell population that was positive for both markers is indicated.

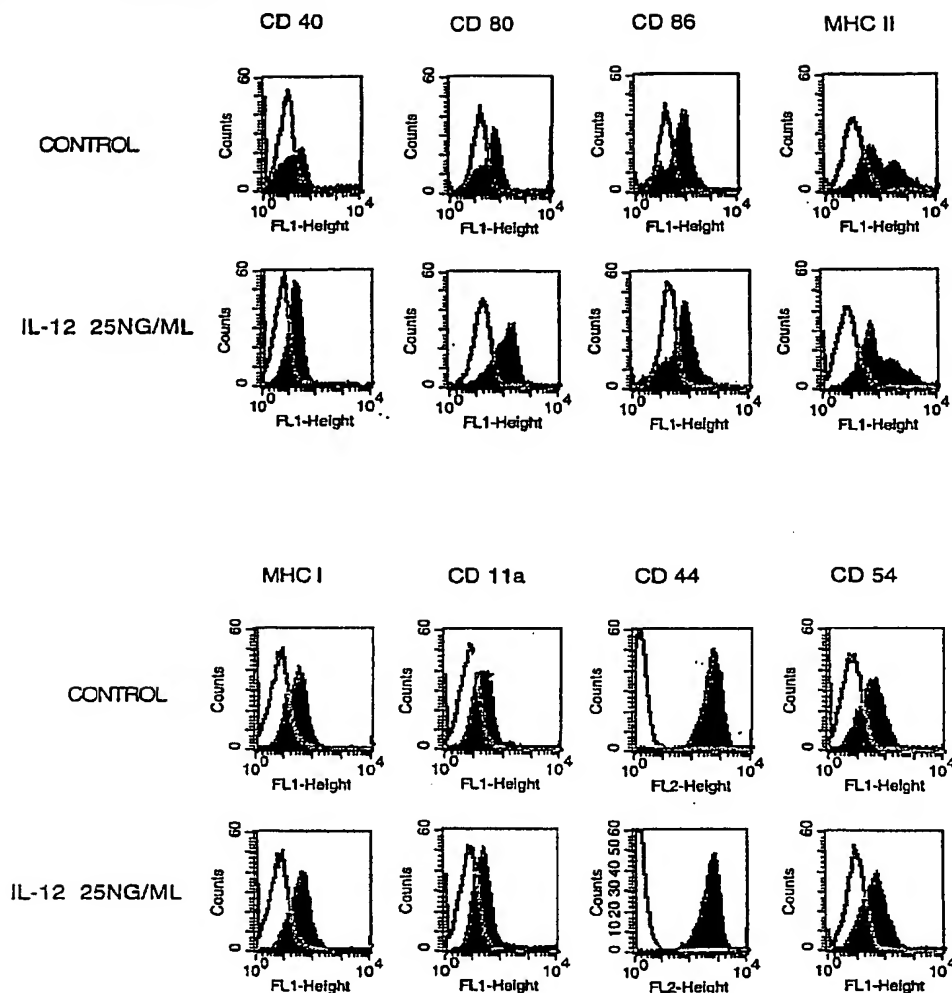


Fig. 2. DC derived from bone marrow progenitors were treated from 3–7 days of culture with medium control or IL-12 at 25 ng/ml. After a total of 10 days of culture DC were labelled with different antibodies. The number of cells (ordinate) with different levels of fluorescent labelling (abscissa) are shown for cells exposed to an isotype control (open histogram) or to a specific antibody (filled histogram). Representative of three experiments performed.

experiments where CD80 expression was elevated there was an increase in the stimulatory capacity of these cells. Results from one experiment where IL-12 treatment at 25 and 50 ng/ml both increased expression of CD80 T cell proliferation are shown in Fig. 3(B). The effect of IL-12 dosimetry is indicated as even in this experiment IL-12 treatment at 50 ng/ml was less effective than IL-12 at 25 ng/ml in T cell stimulation with 1000 DC. IL-12 treatment at 10 ng/ml was associated with a reduction in T cell proliferation in the MLR proliferation but this did not reach statistical significance (Fig. 3B).

Discussion

IL-12 administration at 25 ng/ml to maturing LDC increased the surface expression of CD80 which was associated with an increase in stimulation of T cell proliferation by DC in the MLR. This work also confirms the finding that IL-12 can be secreted by DC in cultures of LDC developing from bone marrow cells (6). The results raise the concept that IL-12 produced by the DC themselves could influence their maturation; slight variability in the level of endogenously

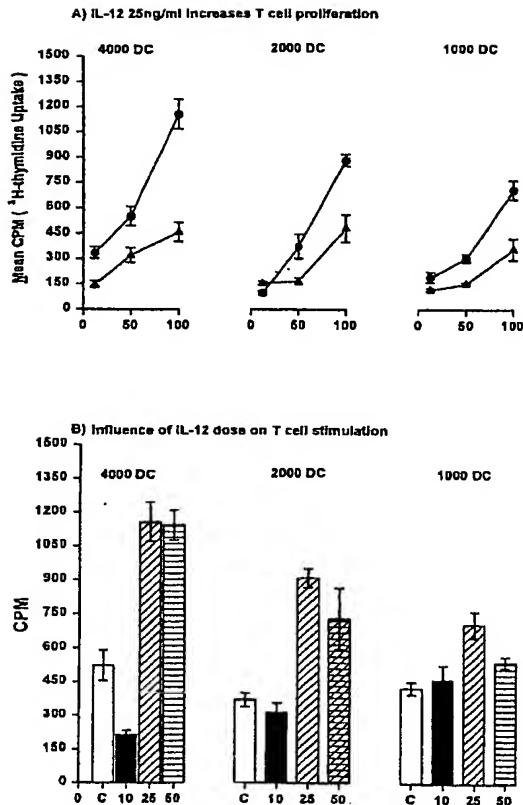


Fig. 3. (A) DC derived from bone marrow progenitors were treated with medium or IL-12 at 25 ng/ml from day 3 to 7 of culture. After a total of 10 days culture DC were incubated with CBA lymph node responder cells in 20 μ l 'hanging drop' cultures. Lymphocyte proliferation was determined from the uptake of [3 H]thymidine for 2 h on day 3 of culture. The graph shows results from one of four similar experiments: Δ , control DC; \bullet , IL-12-treated DC. Lymph node cells alone gave counts of <50 c.p.m. Differences in stimulation of T cell proliferation by control DC and IL-12-treated DC were statistically significant in all experiments ($P < 0.05$). (B) DC were treated with different doses of IL-12 and the MLR were performed as described above. Graph shows the response of 10^5 CBA lymph node responder cells to stimulation by different doses of control DC (C) or DC treated with IL-12 at 10 (10), 25 (25) and 50 (50) ng/ml. Similar responses were observed with lower lymph node cell concentrations. The mean fluorescence for CD80 labelling of DC used in this lymphocyte proliferation assay was control, 52.8; IL-12, 10 ng/ml, 32.5; IL-12, 25 ng/ml, 96.5; and IL-12, 50 ng/ml, 72.9.

produced IL-12 might contribute to the greater variability in the effects of higher doses of added IL-12.

There is conflicting data on whether maturing bone marrow DC secrete IL-12 independently of T cell contact. Heufler and colleagues detected IL-12 p40 and p70 in supernatants of bone marrow-derived DC, whereas Winzler and her associates only detected bioactive IL-12 after antigen presentation to T

cells (6,12). One reason for this discrepancy may well lie in the methods used to generate DC from bone marrow progenitors as DC in this and the Heufler study were more mature than the cells used by Winzler; the ability of DC to produce IL-12 may be proportional to the maturational stage of these cells. This current study shows that IL-12 administration *in vitro* can influence the maturation and immunostimulatory function of DC independently of T cell contact.

IL-12 is likely to act directly on DC since there were few contaminating cells. In addition, DC are the principal stimulatory cells in the MLR and macrophages show little or no activity in this lymphocyte proliferation assay supporting the evidence of an effect directly on DC (13,14). Some of the differences in T cell proliferation observed with the highest doses of IL-12 may again reflect underlying variation in endogenous IL-12 production by DC in different cultures. In addition to any autocrine effects of IL-12 on DC function, the amount of IL-12 that these cells produce may influence the magnitude of T cell proliferation observed in MLR. The mechanisms by which exogenous and endogenous IL-12 may interact to enhance the antigen-presenting capacity of DC remains to be determined.

This work adds IL-12 to the growing numbers of cytokines that can influence antigen presentation by DC. Short-term culture with GM-CSF enhances the stimulatory capacity of skin DC in the MLR (15,16). TNF- α enhances the maturation and allostimulatory capacity of DC in long-term murine bone marrow cultures (12). IL-1 α administration has been shown to enhance T cell proliferation in the MLR by spleen, thymus and skin DC (15,17,18). Since IL-12 is produced by DC, it may have an autocrine effect on DC maturation and function in the MLR; this contrasts with IL-1 α , which is neither produced by DC nor detected in allogeneic MLR (19,20).

Two previous studies had suggested that IL-12 may influence DC antigen-presenting function to T cells, although neither provided any experimental evidence for this. Using BALB/c mice transgenic for V β 11 and V β 3 as a model to examine the development of T $_H$ 1 cells it was proposed that IL-12 and IFN- γ could provide a stimulus to antigen-presenting cells to promote IFN- γ secretion by CD4 lymphocytes (21). Secondly, Enk and colleagues proposed that IL-12 release from human keratinocytes after exposure to allergen could modify antigen presentation by Langerhan cells during primary immune responses in the skin (22).

Changes in cell surface expression of CD80 are known to influence T cell stimulation by DC. Administration of GM-CSF *in vitro* to Langerhans cells and other tissue DC increases their ability to stimulate T cell proliferation in allogeneic MLR via CD80 up-regulation (23). On the other hand, IL-10 can inhibit antigen presentation by Langerhans cells to CD4 T $_H$ 1 lymphocytes in BALB/c mice by suppressing their CD80 expression (24). One possible explanation for the increase in T cell proliferation by IL-12-treated DC is that T $_H$ 1 cells are dependent on CD80 for their continued stimulation, as it has been shown that CD80 synergizes with IL-12 to produce IL-2 receptor in murine T $_H$ 1 clones (25). It needs to be established how IL-12 treatment increases the expression of CD80 on DC. Whether IL-12 acts directly on DC via its receptor and Jak-STAT family of transcription molecules or indirectly

through the secretion of IFN- γ and TNF- α remains to be determined.

The action of IL-12 on DC function may partially explain its mechanism of action in the treatment of infectious diseases and cancer as well as its role in the induction of organ-specific autoimmune diseases. Addition of IL-12 to peripheral blood mononuclear cells obtained from HIV-infected individuals restores the proliferative responses by T cells to a number of different stimuli (26). As DC infection by HIV can lead to reduced T cell proliferation, it has been proposed that IL-12 may be acting on DC to restore their function in this disease (27). The possibility that IL-12 can influence antigen presentation by DC was also suggested by two papers which showed that IL-12 treatment *in vitro* can enhance the ability of DC to induce anti-tumour activity *in vivo* (28,29). IL-12 has also been implicated in the development of autoimmune joint and central nervous system disease (30). Substitution of *Mycobacterium tuberculosis* by IL-12 can induce severe arthritis in DBA/1 mice injected with Freund's incomplete adjuvant and type II collagen, suggesting that IL-12 is acting on antigen-presenting cells (31). Increased expression of IL-12 p40 and CD80 has been described in neuronal plaques of patients with multiple sclerosis, which again suggests that IL-12 may also be acting on antigen-presenting cells in addition to T cells in this disease (32). The amount of endogenous IL-12 production by DC may have contributed to the effects observed with IL-12 in these studies. Localized production of IL-12 by DC may have either additive or synergistic effects on the systemic administration of IL-12. This could be an important contributor to the outcome of IL-12 therapy in infectious diseases and cancer, and susceptibility to autoimmune diseases.

IL-12 has complex effects on haematopoiesis. *In vitro* administration of IL-12 directly increases the numbers of CD34 bone marrow stem cells and their myeloid progeny in synergy with other growth factors such as stem cell factor (SCF) or IL-3 (33). IL-12 *in vivo* decreases peripheral blood counts and bone marrow cellularity largely via IFN- γ induction (34). In this *in vitro* study IL-12 had no significant effects on DC numbers derived from bone marrow progenitors. The differences between this work and other *in vitro* studies may be that IL-12 shows little synergy with GM-CSF in promoting haematopoiesis. Further studies examining IL-12 administration in combination with SCF are needed to determine if this cytokine can alter *in vitro* DC yields from bone marrow-derived precursors.

In conclusion, this study demonstrates that bone marrow-derived DC secrete IL-12 p40 and p70, and that IL-12 administration can alter their phenotype and function. The effect of IL-12 on antigen presentation of DC could be used to enhance the effectiveness of vaccines, and could also underlie some of its therapeutic action in the treatment of infectious diseases and cancer.

Abbreviations

DC	dendritic cell
GM-CSF	granulocyte macrophage colony stimulating factor
LDC	low-density cell population
MLR	mixed lymphocyte reaction

SCF	stem cell factor
TNF	tumor necrosis factor

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